Univerzita Karlova v Praze

1. lékařská fakulta

Studijní program: Fyziologie a patofyziologie člověka

Studijní obor: Fyziologie a patofyziologie člověka

Mgr. Jana Vítků

VLIV VYBRANÝCH ENDOKRINNÍCH DISRUPTORŮ NA LIDSKOU SPERMATOGENEZI

(Polychlorované bifenyly a bisfenol A v mužské reprodukci)

THE IMPACT OF SELECTED ENDOCRINE DISRUPTORS ON HUMAN SPERMATOGENESIS

(Polychlorinated biphenyls and bisphenol A in human reproduction)

Disertační práce

Školitel:

Ing. Martin Hill, DrSc.

Praha, 2015

Identifikační záznam:

VÍTKŮ, Jana. *Vliv vybraných endokrinních disruptorů na lidskou spermatogenezi. [The impact of selected endocrine disruptors on human spermatogenesis].* Praha, 2015. 149 stran, 10 příloh. Disertační práce. Univerzita Karlova v Praze, 1. lékařská fakulta. Endokrinologický ústav. Vedoucí práce: Hill, Martin.

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem řádně uvedla a citovala všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

V Praze, 14. 12. 2015

JANA VÍTKŮ

Obsah

Seznam zkratek

3βHSD	3β-hydroxysteroidní dehydrogenáza
7α-OH-DHEA	7α-hydroxy-dehydroepiandrosteron
7β-OH-DHEA	7β-hydroxy-dehydroepiandrosteron
11βHSD	11β-hydroxysteroidní dehydrogenáza
17-OH-PREG	17α -hydroxy-pregnenolon
17βHSD	17β-hydroxysteroidní dehydrogenáza
ADION	androstendion
AhR	receptor pro arylované uhlovodíky
AR	androgenní receptor
BPA	bisfenol A
cAMP	cyklický adenosin monofosfát
CYP11A1	cytochrom P450scc; cholesterol desmoláza
CYP17A1	17α-hydroxyláza/17,20lyáza
DEHP	diethylhexyl ftalát
DES	diethylstilbestrol
DHEA	dehydroepiandrosteron
DHT	dihydrotestosteron
E1	estron
E2	17β-estradiol
E3	estriol
ED	endokrinní disruptor
EDTA	ethylendiamintetraoctová kyselina
ECHA	European Chemical Agency
ELISA	enzymová imunoanalýza na pevné fázi (enzyme-linked immunosorbent assay)
EPA	Environmental protection agency
ER	estrogenní receptor
FSH	folikuly stimulující hormon
FT	volný testosteron
GnRH	gonadotropin releasing hormone; gonadoliberin
GR	glukokortikoidní receptor
LH	luteinizační hormon
LOD	limit detekce
MAPK	mitogenně aktivovaná protein kináza
PBR	periferní benzodiazepinový receptor
PCB	polychlorované bifenyly
PPARγ	receptor aktivovaný proliferátory peroxisomů γ
PR	progesteronový receptor

PREG	pregnenolon
RIA	radioimunoanalýza
Se	selen
SHBG	pohlavní hormony vázající globulin
StAR	steroidní akutní regulační protein
T	testosteron
TR	thyroidní receptor
UHPLC-MS/MS	vysokoúčinný kapalinový chromatograf s tandemovou hmotnostní detekcí
WMA	World Medical Association; Světová lékařská organizace
Zn	zinek

Souhrn

Steroidní hormony hrají důležitou roli během spermatogeneze, v produkci spermatu a v neposlední řadě také v udržování sekundárních mužských pohlavních znaků a libida. Jsou také diskutovaným cílem pro látky, které se nazývají endokrinní disruptory (ED). Dosud však nebyla provedena žádná studie, která by se zabývala vlivem ED na steroidní spektrum ve 2 biologických matricích – v plasmě a seminální plasmě.

Cílem mé disertační práce bylo vyvinout a zvalidovat metodu pro stanovení bisfenolu A (BPA) a spektra steroidů v plasmě a seminální plasmě a tuto metodu společně s dalšími ukazateli využít k vyhodnocení vlivu BPA a polychlorovaných bifenylů (PCB) na lidskou spermatogenezi a steroidogenezi.

Byly vyvinuty a zvalidovány 2 nové metody na stanovení BPA a 11 steroidů v plasmě a seminální plasmě a tyto metody byly použity ke stanovení 191 vzorků v obou tělních tekutinách u mužů s různým stupněm neplodnosti. Současně byly v plasmě stanoveny hladiny 6 kongenerů PCB, gonadotropinů, selenu a zinku. K vyhodnocení vztahů mezi všemi analyty byly použity parciální korelace adjustované na věk a BMI.

BPA v seminální plasmě negativně koreloval s koncentrací spermií ($r=-0.212$; $p=0.005$), celkovým počtem spermií ($r=-0.178$; $p=0.018$) a jejich morfologií ($r=-0.156$; $p=0.049$). Tyto vztahy byly zjištěny jen pro BPA v seminální plasmě, což poukazuje na unikátnost této tělní tekutiny ve studiu ED. V seminální plasmě BPA negativně koreloval s hladinami steroidních prekurzorů (pregnenolon, 17α-hydroxy-pregnenolon), opačné vztahy byly zaznamenány pro BPA a steroidní prekurzory v krevní plasmě. V obou tělních tekutinách byly zjištěny pozitivní korelace BPA s hladinami estrogenů. Součet hladin kongenerů PCB negativně koreloval s koncentrací testosteronu, volného testosteronu a dihydrotestosteronu v plasmě.

BPA negativně přispívá k výsledné kvalitě spermií. Výsledky naznačují odlišný vliv BPA na gonadální a adrenální steroidogenezi a následné narušení steroidogeneze i v dalších krocích. Hladiny PCB v životním prostředí u studované skupiny mužů překvapivě neměly vliv na kvalitu spermií, ale snižovaly koncentrace androgenů v plasmě.

Summary

Steroid hormones in testis play an important role in spermatogenesis, maintenance of the male reproductive tract, production of semen and the maintenance of secondary sex characteristics and libido. They are also discussed as a target for substances called endocrine disruptors (EDs). No complex study was conducted on evaluation of relationships between EDs and steroid spectrum in 2 biological fluids; seminal plasma and plasma.

The aim of the PhD. thesis was to develop and validate a method for determination of bisphenol A (BPA) and steroid spectrum in plasma and seminal plasma and to shed more light into mechanisms of ED action and effects of BPA and polychlorinated biphenyls (PCBs) on human spermatogenesis and steroidogenesis.

Two new liquid-chromatography mass spectrometry methods for determination of BPA and 11 steroids in plasma and seminal plasma were developed and validated. The methods were used for estimation of analyte concentrations in 191 men with a different degree of fertility. Concurrently, the levels of six congeners of PCBs, gonadotropins, selenium and zinc in plasma were estimated. Partial correlations adjusted for age and BMI were calculated to evaluate relationships between these analytes.

Seminal BPA, but not plasma BPA, was negatively associated with sperm concentration ($r=-0.212$; $p=0.005$), sperm count ($r=-0.178$; $p=0.018$) and morphology ($r=-0.156$; $p=0.049$). These relationships were observed only in seminal plasma BPA, which indicates the uniqueness of seminal plasma in the ED research. BPA in seminal plasma negatively correlated with seminal steroid precursors (pregnenolone, 17α-hydroxy-pregnenolone), on the other hand, the opposite results were found for BPA and steroid precursors in plasma. The sum of PCB congeners was negatively associated with testosterone, free testosterone and dihydrotestosterone in plasma.

BPA negatively contributes to the final state of sperm quality. Moreover, the present data indicate that BPA influence human gonadal and adrenal steroidogenesis at various steps. Environmental levels of PCBs in our study population negatively correlated with androgen levels, but surprisingly without negative effects on sperm quality.

1 Úvod

V posledních dekádách se do životního prostředí dostávají antropogenní činností tisíce tun různých chemikálií. O těchto látkách se předpokládalo, že nemají žádnou, či mají jen velmi malou biologickou toxicitu. Dnes nás obklopují běžně v každodenním životě, jsou např. ve vzduchu, vodě, půdě, potravinách, elektronice či plastech a nyní se ukázalo, že mnoho z nich má schopnost zasahovat do endokrinního systému. Tyto látky se nazývají endokrinní disruptory (ED). Postupně přibývají důkazy o jejich negativních vlivech jak na živočišnou populaci, tak na lidský organismus [1, 2].

V posledních desetiletích byly také zaznamenány zhoršující se reprodukční funkce u lidí i zvířat. Již v roce 1992 byla publikována rozsáhlá metaanalýza, kde bylo prezentováno postupné snižování počtu spermií u mužů z různých částí světa v průběhu uplynulých padesáti let [3]. Tyto výsledky podpořily i další studie [4-6], naopak některé pokles kvality spermií neprokázaly [7, 8]. Byl zjištěn také sekulární pokles hladin testosteronu u mužů [9, 10].

Carlsenová [3] svoje analýzy dávala do souvislosti právě se zvyšujícím se výskytem chemikálií v prostředí. Kromě kvality spermií byl zaznamenán vyšší výskyt nádorů varlat [11] a vyšší riziko kryptorchismu a hypospadie [12, 13]. Stále častěji se uvažuje, že expozice ED je největší viník těchto problémů. Mezi nejdiskutovanější ED patří polychlorované bifenyly (PCB), dioxiny, ftaláty, a bisfenol A (BPA), a to buď kvůli jejich perzistenci v prostředí a schopnosti bioakumulaci v potravinovém řetězci (PCB, dioxiny), nebo kvůli jejich všudypřítomnosti, např. v obalech na potraviny (BPA, ftaláty).

Lidé jsou vystaveni vlivu ED ve svém každodenním životě a není možné zcela zabránit jejich působení. Hlavními cestami, jak se ED dostanou do organismu, je požití kontaminované potravy a tekutiny, dýchání kontaminovaného vzduchu, či transdermálně [14].

V roce 2015 byla publikována rozsáhlá studie, v níž byla skupina vědců vyzvána, aby na základně dostupných údajů z Evropské unie odhadli ekonomickou zátěž a náklady spojené s onemocněními či poruchami, které mohou být připsány ED [15]. Na základě publikovaných článků z oblasti reprodukce byly vyhodnoceno mírné epidemiologické a silné toxikologické riziko mužské neplodnosti spojené s expozicí ftalátům, které si se 40-69% pravděpodobností vynutí dalších 618000 procedur v oblasti

asistované reprodukce. Tyto procedury stojí EU 4.7 miliardy eur. Příslušné odhady byly zaměřeny jen na expozici ftalátům a polybromovaným diphenyl etherům, protože jejich vliv na volně žijící zvířata i lidskou populaci je nejlépe dokumentován [16].

Naopak některé látky přítomné v životním prostředí mohou, pokud jde o mužskou reprodukci, působit příznivě. Mezi ně patří selen a také zinek. Selen (Se) je klíčovým prvkem pro fyziologický vývoj varlat a neporušenou spermatogenezi [17]. Některé studie prokázaly u mužů s deficitem Se zlepšení motility spermií a zvýšení šance k početí po jeho suplementaci [18]. Obsah zinku (Zn) ve varlatech a v seminální tekutině je vyšší než v ostatních tkáních těla. Zinek má antioxidační vlastnosti a hraje roli v odstraňování reaktivních kyslíkových radikálů. Výsledky několika studií naznačují, že snížení koncentrace Zn v seminální tekutině je rizikový faktor pro fyziologickou tvorbu spermií a jednou z možných příčin poruch mužské plodnosti [19, 20].

Ačkoli účinky některých ED na organismus jsou dobře známé, u jiných stále převládá nejistota a nekonzistence. Cílem disertační práce bylo přispět k objasnění vlivu některých vybraných faktorů vnějšího prostředí na lidskou spermatogenezi a steroidogenezi.

2 Pracovní hypotéza

Jedním z možných mechanismů působení ED by mohlo být ovlivnění aktivity významného enzymu metabolismu steroidů, 11β-hydroxysteroidní dehydrogenázy (11βHSD) typu 1 a 2. Isoformy tohoto enzymu chrání testes před nadbytkem glukokortikoidů tak, že upravují lokální poměr kortizolu ke kortizonu ve prospěch kortizonu. Pokud by tento enzym byl inhibován, nadbytečné množství kortizolu v Leydigových buňkách způsobí snížení tvorby testosteronu, který je zásadní pro fyziologickou tvorbu spermií. Dalším důležitým faktorem pro celkový počet, motilitu a morfologii spermií je přiměřená koncentrace některých prvků, konkrétně Zn nebo Se. Všechny tyto vlivy, jak positivní (Se a Zn) tak negativní (vysoké hladiny glukokortikoidů a působení endokrinních disruptorů) mají podle naší hypotézy vliv na normospermii.

3 Cíle disertační práce

Cílem disertační práce je přispět k objasnění vlivu některých vybraných faktorů vnějšího prostředí na lidskou spermatogenezi, se zaměřením na steroidy a jejich úlohu v mužské reprodukci. Vytyčené cíle jsou konkrétně tyto:

1) Vyvinout metodu na stanovení steroidů a BPA v plasmě a seminální plasmě na vysokoúčinném kapalinovém chromatografu s tandemovou hmotnostní detekcí (UHPLC-MS/MS)
2) Vyšetřit rozdíly v hladinách vybraných ED, steroidů a dalších relevantních analytů u 4 skupin mužů s různým stupněm neplodnosti
3) Zhodnotit vliv vybraných ED na parametry spermiogramu
4) Zhodnotit vliv vybraných ED na aktivitu 11βHSD a dalších enzymů steroidogeneze.
5) Zhodnotit vliv vybraných ED na hladiny imunoaktivních a dalších steroidů.
6) Zhodnotit vliv vybraných ED na hladiny vybraných stopových prvků
7) Zhodnotit, nakolik spolu korelují jednotlivé analyty v plasmě a seminální plasmě

4 Literární přehled

4.1 Obecné vlastnosti endokrinních disruptorů

Endokrinním disruptorem je jakákoliv látka, která nějakým způsobem ovlivňuje endokrinní systém. Přesná definice podle Environmental Protection Agency (EPA) definuje ED jako exogenní látku, která zasahuje do syntézy, sekrece, transportu, vazby, akce nebo eliminace přirozených hormonů zodpovědných za udržování homeostázy, reprodukci, vývoj a/nebo chování. Dnes pod tento pojem spadá několik tisíc látek.

Disruptory jsou různorodá skupina látek, které vykazují některé zajímavé charakteristiky. Podobně jako hormony mohou účinkovat ve velice malém množství. Nižší hladiny ED mohou mít ve výsledku větší účinky na cílovou tkáň než vyšší hladiny ED. Mohou také vykazovat netradiční dynamiku dávky a odpovědi, kdy křivky závislosti dávky na odpovědi mohou mít tvar U či invertované U [1]. Počet publikací o ED dramaticky narůstá – nejvíce zájmu přitahuje jejich vztah k funkci štítné žlázy, na druhém místě je fertilita a na dalším pak onkogenní působení, vztah k nástupu a průběhu puberty a nejnověji pak vztah k obezitě, kdy je některými autory připisován ED klíčový vztah k pandemii obezity.

Důležitý v případě disruptorů je věk v době expozice. Vystavení se působení ED v dospělosti může mít zcela odlišné důsledky než expozice během vývoje. U dospělých je zpravidla potřeba vyšší hladina ED, aby působila toxicky na organismus. Naopak během vývoje organismu stačí nízká dávka po kratší dobu a může mít trvalé následky až do dospělosti, kdy už ED dávno v těle není přítomen. Tento koncept je pojmenován „the fetal basis of adult disease“ neboli expozice ED při vývoji organismu je základ nemoci/poruchy v dospělosti [21]. Z řady možných mechanismů zásahu ED do systému reprodukčních funkcí jsou nejdůležitější modifikace metabolismu hormonů, modifikace jejich působení na příslušné receptory a epigenetické vlivy. Vedle rozdílného působení ED v různých vývojových obdobích člověka, zvláště intenzivních ve „vývojových oknech“ (intrauterinně, v raném dětství, v pubertě a ve stáří) patří ke zvláštnostem působení ED také latence působení, někdy i transgenerační působení směsí (častý synergismus) a netradiční závislosti na dávce. Příkladem synergického působení je dieldrin a endosulfan, které působí 100x vyšším estrogenním efektem v kombinaci než

samostatně - EC50 = 100nM [22]. Podobně dieldrin působí synergicky i s jinými disruptory (lindan) [23]. Jiní autoři [24] prokazují, že kooperativní účinek dieldrinu a endosulfanu, pokud vůbec existuje, není natolik silný, aby hrál větší roli v ohrožování lidského zdraví.

Do mužských reprodukčních funkcí mohou zasahovat zejména ED s vlastnostmi anti-androgenů nebo estrogenů. U mužů byla prokazována spoluúčast ED na řadě klinických poruch jako je snížená kvalita spermií, karcinom zárodečných buněk, anomálie mužské reprodukční soustavy, karcinom prostaty a erektilní dysfunkce [1, 25, 26].

Nejznámějšími látkami, které zasahují do endokrinního systému, jsou polychlorované bifenyly (PCB), bisfenol A (BPA), dioxiny, ftalátové estery, fytoestrogeny a pesticidy. Mnohé z nich jsou velmi perzistentní (PCB, dioxiny), což má za následek jejich bioakumulaci v potravním řetězci a také v lidském organismu, jiné se naopak rychle rozkládají a mohou tak působit jen po omezenou dobu (ftaláty). Přesto ve vývoji a dozrávání varlat existuje několik kritických úseků, které jsou k expozici disruptorům obzvláště citlivé.

4.2 Vlastnosti vybraných endokrinních disruptorů

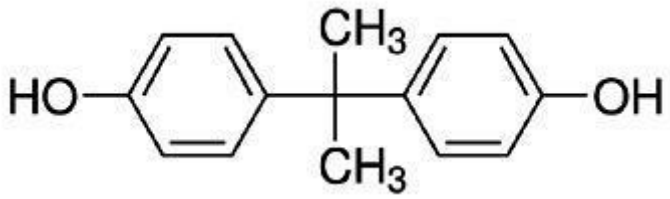
Mezi ED vybrané pro výzkum v rámci disertační práce byl zařazen BPA a 6 kongenerů PCB (28, 52, 101, 138, 153 a 180).

4.2.1 Bisfenol A

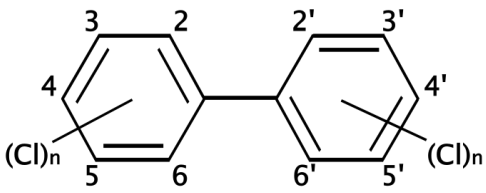
Bisfenol A (Obr. 1) patří k vůbec nejrozšířenějším ED. Je obsažen hlavně v plastech, dále pak např. epoxidových pryskyřicích, kterými bývá potažen vnitřek plechových nádob, v dentálních výplních či ve stvrzenkách z pokladen [27]. Poprvé byl syntetizován v roce 1891 a ve 30. letech minulého století byl prověřován během hledání syntetických estrogenů pro lékařské účely. Estrogenicita BPA byla potvrzena, a ještě vyšší estrogenicita byla zjištěna u diethylstilbestrolu (DES). Ve 40. a 50. letech se pro BPA tedy našlo jiné uplatnění – stavební kámen pro výrobu polykarbonátových plastových hmot a jako aditivum do jiných plastových hmot [28].

Molekuly BPA jsou v plastech spojené esterovou vazbou, která je předmětem hydrolýzy hlavně při vyšších teplotách nebo v kyselém či zásaditém prostředí [27, 29-31], což má za následek uvolňování BPA z výrobků. Účinky BPA v organismu byly předmětem zájmu mnoha studií. Kromě působení na klasických estrogenních receptorech (ER) α i β [32-34] BPA interaguje i s estrogenům příbuzným receptorem γ [35, 36], androgenním receptorem (AR) [37, 38], thyroidním receptorem (TR) [39], glukokortikoidním receptorem (GR) [40], receptorem aktivovaným proliferátory peroxisomů γ (PPARγ) [41, 42], pregnanovým X receptorem [43] nebo může působit na neklasické membránové ER [44]. Tyto účinky byly pozorovány při koncentracích srovnatelných s koncentracemi endogenních hormonů [27].

Obr. 1 Chemická struktura BPA



Obr. 2 Chemická struktura PCB



4.2.2 Polychlorované bifenyly

Polychlorované bifenyly (PCB) (Obr. 2) jsou z chemického hlediska chlorderiváty dibenzenu. Tato skupina zahrnuje přes 200 kongenerů neboli příbuzných látek, které měly široké využití: tekutiny do chladících zařízení (izolace či chlazení), zpomalovače hoření, aditiva do pesticidů či barviv [45]. PCB jsou rozpustné v tucích, a proto se také akumulují v tukové tkáni. V 70. letech minulého století byly kvůli své toxicitě zakázány, ale vzhledem k jejich perzistenci a jejich dříve hojném využívání jsou stále všudypřítomné v prostředí. Lidé je přijímají hlavně potravou, protože se bioakumulují v potravním řetězci. Epidemiologické studie ukazují na nepříznivé účinky PCB hlavně na motilitu spermií. Tento vztah byl konzistentně zjištěn ve studiích nejen z Ameriky, ale i dalších jako například v Indii, Nizozemí, na Taiwanu, Švédsku [46-49]. Další studie vyšetřovaly vztah mezi PCB a reprodukčními hormony [48, 50-54], zde již výsledky ovšem nedosahovaly takové konzistence.

4.3 Spermatogeneze

Spermatogeneze je složitý proces, při kterém nezralá zárodečná buňka podstupuje dělení, diferenciaci a meiosu, aby se stala haploidní spermií. Zahrnuje 3 fáze: spermatogoniální fázi, fázi spermatocytů a fázi spermatidovou [55]. V první fázi spermatogonie, relativně nespecializované diploidní zárodečné buňky, ležící na vnitřní straně semenotvorných kanálků [56], podstupují mitózu za vzniku diploidních spermatocytů. V další fázi diploidní spermatocyty projdou dvěma meiotickými děleními, během kterých vzniknou haploidní spermatidy. Z nezralých kulatých spermatid se v poslední fázi, nazývané spermiogeneze, stávají zralé spermie [57].

Pro spermatogenezi jsou zásadní Sertoliho buňky, které nasedají na bazální membránu v semenotvorných kanálcích. Mezi sebou jsou spojeny těsnými spojeními neboli „tight junctions“ a tvoří tak bariéru mezi krevním řečištěm a semenotvornými kanálky [58]. Sertoliho buňky podporují zrání zárodečných buněk buď přímým kontaktem se zárodečnou buňkou nebo řízením vnitřního prostředí v semenotvorných kanálcích [59]. Regulovány jsou folikuly stimulujícím hormonem (FSH) z adenohypofýzy a testosteronem, který je produkován Leydigovými buňkami varlat jako odpověď na stimulaci luteinizačním hormonem (LH) z adenohypofýzy [55]. Testosteron působí na Sertoliho buňky prostřednictvím androgenního receptoru a stimuluje syntézu různých proteinů a trofických faktorů ve specifických fázích spermatogeneze [60]. Mezi trofické faktory parakrinně secernované Sertoliho buňkami patří např. insulin-like growth factor 1 (IGF-1), nerve growth factor (NGF), growth factor derived from glia (GDNF) a stem cell factor (SCF) [61].

Proces spermatogeneze začíná pod vlivem steroidních androgenů a FSH v pubertě a pokračuje v průběhu celého života. Proto je muž během celého tohoto období citlivý na změny v životním stylu a také na vystavení se různým chemikáliím v životním prostředí [62]. Neméně důležité je období fetálního vývoje, kdy jsou položeny základy mužských pohlavních orgánů. Změny ve vývoji způsobené ED by mohly mít zásadní vliv na kvalitu spermatogeneze v dospělosti [62].

4.4 Steroidogeneze v Leydigových buňkách

Steroidogeneze v Leydigových buňkách je řízena LH. Po vazbě LH na receptor spojený s G-proteiny je stimulována tvorba cyklického adenosin monofosfátu (cAMP) [63]. Vyšší hladiny cAMP následně aktivují na cAMP závislou proteinkinázu A (PKA), která fosforyluje seriny na steroidním akutním regulačním proteinu (StAR), čímž ho aktivuje. [64]. StAR společně s periferním typem benzodiazepinového receptoru (PBR) zprostředkovávají přenos cholesterolu od vnější k vnitřní mitochondriální membráně [65-67]. Přenos cholesterolu je prvním a zároveň limitujícím krokem ve steroidogenezi. Jakmile se cholesterol dostane do vnitřní mitochondriální membrány, je konvertován na pregnenolon cytochromem P450scc (CYP11A1). Pregnenolon se pak z mitochondrie přesouvá do hladkého endoplasmatického retikula, kde je pomocí 17α-hydroxylázy/17,20lyázy (CYP17A1) konvertován přes 17α-hydroxy-pregnenolon (17-OH-PREG) na dehydroepiandrosteron (DHEA). V dalším kroku se uplatní enzym 3β-hydroxysteroidní dehydrogenáza (3βHSD), která katalyzuje přeměnu DHEA na androstendion (ADION) a poslední fáze, přeměna na testosteron (T), je zajištěna 17β-hydroxysteroidní dehydrogenázou (17βHSD) typu 3 [68].

4.4.1 Řízení steroidogeneze

Celá signalizační osa je řízena z hypothalamu pulzně vylučovaným gonadotropin-uvolňujícím hormonem = gonadoliberinem (GnRH), který dále působí na adenohypofýzu. Z té se po podnětu vylučují FSH a LH, které se krevním řečištěm dostávají do varlat. LH působí na Leydigovy buňky v intersticiu a stimuluje tvorbu testosteronu, zatímco FSH působí přímo na Sertoliho buňky. Uvolňování GnRH je řízeno hypothalamickým peptidem kisspeptinem-1 a jeho receptorem spřaženým s G-proteiny. Kisspeptin je kódován genem *KISS1*, který je cílem gonadálních steroidů u obou pohlaví [69]. Kisspeptinové neurony exprimují ERα, progesteronový receptor (PR) a AR [70]. Je pravděpodobné, že regulace exprese *KISS1* genu bude fungovat jako negativní zpětná vazba [71]. Dokázáno to bylo např. na samicích myší, kdy po ovariektomii se gen *KISS1* exprimoval více a jeho exprese po podání estradiolu zase klesla [72]. U myší a lidí, kterým chybí kisspeptinový receptor, se objevuje hypogonadotropní hypogonadismus a s tím související infertilita [73, 74].

Dalšími hormony podílející se na řízení osy hypothalamus-hypofýza-testes jsou inhibiny a aktiviny. Nejprozkoumanějším inhibinem je inhibin B tvořící se v Sertoliho buňkách v testes, který inhibuje tvorbu FSH v hypofýze [75-77]. Opačné účinky má aktivin A produkovaný testes, hypothalamem i hypofýzou, který tvorbu FSH stimuluje [78, 79]. Anti-Mülleriánský hormon je dalším členem rodiny transforming growth factor β (TGFβ), který se tvoří v Sertoliho buňkách a podílí se na regulaci steroidogeneze [80].

4.5 Mechanismy působení ED

4.5.1 Kompetice o vazbu na receptory

Nejdéle známým mechanismem působení ED je jejich vazba na jaderné receptory. Endokrinní disruptory jsou strukturně podobné mnoha steroidům, účinkují také v nízkých dávkách a mohou mít lipofilní vlastnosti. Proto jsou schopné napodobovat endogenní hormony a napodobovat jejich mechanismus působení, transport a ukládání ve tkáních. Jejich vlastnosti jim umožňují navázat se na jaderné receptory a tím aktivovat nebo potlačovat jejich funkci. Je známé působení ED na ERα i β [32-34], AR [81] , TR [39, 82], PR [83], retinoidní receptor [84] receptor pro arylované uhlovodíky (AhR) [85, 86] , GR [40] či PPARγ [87-90] [91] či pregnanový X receptor [92].

Po navázání ED na receptor se komplex přesune do jádra, má však často jiné účinky než přirozený ligand. Pokusy s estradiolem a DES ukázaly, že DES jakožto syntetický estrogen se sice naváže na ER stejně jako estradiol, ale některé geny se pod jeho působením exprimují více a jiné naopak méně, takže výsledný efekt těchto dvou látek je rozdílný [93]. Bylo prokázáno, že ED se mohou vázat nejen na jaderné receptory ale i na membránové ER [94, 95] či na nesteroidní receptory [1].

4.5.2 Modulace enzymových systémů

Původně bylo působení endokrinních disruptorů vysvětlováno jen vazbou na nukleární steroidní receptory. Dnes již víme, že mohou působit i na jiných úrovních, mimo jiné mohou narušovat steroidní biosyntézu a metabolismus [1] či antioxidačními mechanismy [96]. Negativní účinky disruptorů byly pozorovány v *in vitro* i *in vivo* studiích.

4.5.2.1 Modulace steroidní biosyntézy a metabolismu

Ovlivnění steroidogeneze ED můžeme představit na příkladu ftalátů. Ftaláty ovlivňují steroidogenezi v Leydigových buňkách v závislosti na stadiu fetálního vývoje. Podávání diethylhexyl ftalátu (DEHP) potkanům dva týdny postnatálně má za následek snížení aktivity 17βHSD a hladin T v Leydigových buňkách. Na druhou stranu podávání DEHP dospělým potkanům steroidogenezi v Leydigových buňkách neovlivňuje [97].

V případě podávání xenoestrogenu bisfenolu A byla zjištěna snížená aktivita cytochromu CYP17A1 [98] a stejně tak StAR, CYP11A1, a 17βHSD [99].

Působením na steroidogenní enzymy ED ovlivňují syntézu T Leydigovými buňkami v průběhu fetálního vývoje i v dospělosti. Nejčastějším cílem ED jsou tedy geny kódující StAR, CYP11A1 a CYP17A1, které jsou potřebné pro biosyntézu steroidních hormonů. Následkem změn ve steroidním spektru, které dále ovlivňují zpětnovazebné systémy na ose hypothalamus-hypofýza-gonády, dochází k reprodukční dysfunkci [100].

4.5.2.2 Indukce oxidačního stresu

Stále více důkazů naznačuje, že chemikálie z prostředí mohou snižovat testikulární funkce také narušením rovnováhy mezi pro-oxidačními a antioxidačními mechanismy, čímž aktivují další dráhy, např. dráhu vedoucí k apoptóze. Ačkoliv fyziologické hladiny reaktivních kyslíkových radikálů a apoptózy jsou nutné pro normální funkce varlat, patologické hladiny mohou být škodlivé [101].

Ve varlatech je vyvinuta řada antioxidačních mechanismů, mezi nimiž působí jak enzymatické tak neenzymatické složky [102]. Mezi antioxidační enzymy chránící varlata patří např. superoxid dismutáza, glutathion peroxidáza a kataláza. Superoxid dismutáza nejprve konvertuje superoxidový anion na peroxid vodíku, který pak kataláza a superoxid dismutáza převede na vodu.

Několik studií publikovalo výsledky ohledně BPA, ve kterých zvyšuje oxidační stres ve varlatech, nadvarlatech a spermiích u různých živočišných druhů. Podávání BPA potkanům způsobuje snížení motility i počtu spermií v epididymis, dále snižuje aktivitu superoxid dismutázy, katalázy, glutathion reduktázy a glutathion peroxidázy a

s tím související zvýšení hladin peroxidu vodíku a lipidové peroxidace [96]. Ve varlatech byly u myší zjištěny snížené hodnoty redukovaného glutathionu po podání BPA [103].

U endokrinních disruptorů, jako jsou např. BPA, 2,3,7,8-tetrachlorodibenzo-p-dioxin či kadmium, je známo, že indukují oxidativní stres ve varlatech down-regulací antioxidačních enzymů [96, 104, 105]. Nedávné studie ukázaly, že ED mohou způsobit mužskou infertilitu také narušením buněčných spojení mezi Sertoliho a zárodečnou buňkou v testes a mezi Sertoliho buňkami navzájem. K takovému porušení integrity v testes může docházet buď přes signalizační dráhu mitogenně aktivované protein kinázy (MAPK) nebo přes kaskádu fosfatidylinositol-3-kinázy/c-Src/focal adhesion kinázy [106]. Narušení spojení mezi jednotlivými buňkami vede následně k dysregulaci spermatogeneze. Bisphenol A je jedním z disruptorů, které mohou působit přes MAPK dráhu a narušovat spojení „tight junctions“ mezi Sertoliho buňkami v hematotestikulární bariéře [107].

4.5.3 Epigenetické působení ED

Všechny doposud jmenované mechanismy vysvětlují přímé působení ED na organismus. Relativně nedávno bylo zjištěno, že vývoj zárodečných buněk může být ovlivněn také nepřímo, zděděným epigenetickým působením ED. Epigenetické změny jsou takové změny v genové expresi, při kterých DNA sekvence zůstává zachována, ale upravená může být např. metylace DNA, acetylace histonů či microRNA [108]. Dostupné informace z modelů na zvířatech ukazují, že při expozici xenobiotikům v kritických obdobích savčího vývoje může docházet k trvalým a dědičným změnám v epigenetickém stavu. Poprvé byly epigenetické změny v zárodečné linii prezentovány u potkanů Anwayem a kol. [109]. Březí samice potkanů byly vystaveny antiandrogennímu fungicidu vinclozolinu či estrogennímu insekticidu methoxychloru. V F1 generaci byl pozorován snížený počet a viabilita spermií. Tyto účinky byly pozorovány ve všech zkoumaných generacích (až do F4) a korelovaly se změnami v metylaci DNA [109]. Další studie prokázaly hypometylaci DNA při prenatální expozici BPA u potkanů [110] či negativní korelaci mezi hypometylací DNA a hladinami persistentních organických polutantů (např. DDT, p,p´-DDE) v séru [111].

4.6 Legislativa endokrinních disruptorů

Pojem endokrinní disruptor byl poprvé definován na konferenci vědců ve Wingspreadu ve státě Wisconsin v roce 1991: „Mnoho látek uvedených do prostředí lidskou činností je schopných narušovat endokrinní systém zvířat, včetně ryb, volně žijících živočichů a člověka.“ Od té doby se začala řešit problematika ED a jejich potenciální nepříznivé účinky na člověka.

Většina zákonů o environmentálních kontaminantech ve Spojených státech amerických jsou připravovány agenturou EPA, která identifikovala asi 87 000 chemikálií, které jsou komerčně využívány. O drtivé většině z těchto látek máme jen málo informací o jejich toxicitě. Proto v roce 1998 EPA navrhla 2-pilířový postup na testování toxicity chemikálií. První pilíř zahrnuje skríningovou baterii *in vitro* a *in vivo* testů navrženou pro testování potenciálních interakcí látek s endokrinním systémem. Ty látky, které budou vyhodnoceny jako potenciálně interagující, postoupí do druhého pilíře pro určení nežádoucích účinků na organismus a určení dynamiky dávky a odpovědi. Výsledky testů z druhého pilíře pak poslouží k posouzení nebezpečnosti látek, které povedou k regulaci těchto látek na trhu.

V Evropské Unii je hlavním regulačním orgánem ECHA (European Chemical Agency), která se stará o tvorbu a uplatňování právních předpisů o chemických látkách, které vedou k ochraně lidského zdraví a prostředí. V roce 2007 vstoupila v platnost směrnice REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals). Ta zjednodušuje a zlepšuje dřívější legislativní rámec pro chemické látky v Evropské unii a činí průmysl odpovědným za stanovení a vyhodnocení rizik chemikálií a uživatelům musí poskytnout informace o látkách, které vzbuzují mimořádné obavy. Na kandidátské listině látek vzbuzujících mimořádné obavy figuruje v současnosti 144 chemikálií. V květnu minulého roku skončila druhá vlna registrace chemikálií, které jsou dovážené jednou společností do Evropské Unie v množstvích 100 až 1000 tun ručně. Poslední vlna registrace má proběhnout do konce května 2018, kdy mají být zaregistrovány chemikálie dovážející se do EU v množství menší než 1 tuna ročně.

Téma endokrinních disruptorů je v poslední době velmi diskutované. V červnu roku 2013 podepsalo 89 předních vědců z celého světa Berlaymontskou deklaraci o endokrinních disruptorech. V té vyjádřili svůj názor k tomuto důležitému tématu a vyzvali

Evropskou komisi k zavedení regulačních opatření, která jsou v souladu se současnými nejnovějšími vědeckými poznatky a metodami. Podobné stanovisko zaujímá Evropský parlament, který jednomyslně schválil zprávu europoslankyně Åsy Westlund o ochraně veřejného zdraví před endokrinními disruptory. Ta vyzývá k přijetí opatření, která by byla zaměřena na snížení krátkodobé a dlouhodobé expozice osob endokrinním disruptorům. Navrhuje také daleko více se zaměřit na výzkum, který by zlepšil vědecké poznatky o vlivu látek s negativním působením na endokrinní systém a lidské zdraví.

5 Materiál a metodika

5.1 Účastníci studie

Studovaná skupina se skládala ze 191 mužů, kteří navštěvovali Centrum asistované reprodukce Pronatal v období od dubna 2012 do září 2015. Muži se lišili ve stupni plodnosti. Část souboru zahrnovala muže-normospermiky, kde příčina neplodnosti byla na straně partnerky. Ostatní pacienti měli různé defekty spermií od mírných defektů až po azoospermiky. Každý pacient podstoupil standardizované vyšetření ejakulátu (spermiogram), které probíhalo podle kritérií Světové zdravotnické organizace (WHO) z roku 2010. Všichni pacienti podstoupili základní urologické a andrologické vyšetření včetně ultrasonografie prostaty, seminálních váčků a varlat, kde nebyly zjištěny žádné patologické nálezy. Dále byla zaznamenána jejich váha a výška.

Muži byli rozděleni na základě výsledků spermiogramu do 4 skupin. První skupina (n=89) zahrnovala muže s normospermií, do druhé (n=59) byli zařazeni muži s oligospermií, asthenospermií a oligoasthenospermií, třetí skupina (n=25) obsahovala muže s teratospermií, oligoasthenoteratospermií či oligoteratospermií a v poslední skupině byli muži-azoospermici (n=18). Tyto skupiny jsme pojmenovali jako zdravé muže (1.sk), a dále mírně (2.sk), středně (3.sk) a těžce (4.sk) neplodné muže.

Studie byla vedena v souladu s Helsinskou deklarací vydanou Světovou lékařskou asociací (WMA). Protokol byl schválen etickou komisí Endokrinologického ústavu. Každý pacient podepsal informovaný souhlas před zařazením do studie a odběrem biologického materiálu.

5.2 Sběr vzorků

Od každého pacienta byl odebrán vzorek krve do zkumavek pro nesrážlivou krev obsahující ethylendiamintetraoctovou kyselinu (EDTA) a vzorek ejakulátu. Plasma byla získána 10-ti minutovou centrifugací při 1500g. Ejakulát byl centrifugován 10 minut při 2000g a supernatant - seminální plasma - byla následně převedena do nové zkumavky. Vzorky byly uloženy v teplotě -20°C a rezervní vzorky při -80°C až do doby, kdy byly zpracovány. Všechny kroky v pracovním postupu včetně skladování byly provedeny ve skle a za užívání skleněných pomůcek, abychom se vyhnuli kontaminaci BPA. Doba

stání krve v odběrové zkumavce byla snížena na minimum a bylo ověřeno, že touto cestou nedochází ke kontaminaci. Více se tímto problémem zabýváme ve článku Vítků a kol. 2015a [112] (Příloha VIII).

5.3 Roztoky a chemikálie

Kortizol, kortizon a dehydroepiandrosteron (DHEA) byly zakoupeny v Koch-Light Laboratories Ltd. (Colnbrook, Velká Británie); 7α-hydroxy-DHEA (7α-OH-DHEA), 7β-hydroxy-DHEA (7β-OH-DHEA), 7-oxo-DHEA, T, ADION, PREG, 17-OH-PREG, estron (E1), 17β-estradiol (E2), estriol (E3) a deuterované standardy DHEA (d3-DHEA), ADION (d7-ADION), PREG (d4-PREG), 17-OH-PREG (d3-17-OH-PREG), E1 (d4-E1), E3 (d2-E3) s dihydrotestosteronu (d3-DHT) byly od Steraloids (Newport, RI, USA). D4-Kortizol zakoupen v CDN isotopes (Ponte-Claire, Canada). D1-7α-OH-DHEA and d1-7-oxo-DHEA byly získány z Betulinines (Stříbrná Skalice, Česká Republika). D1-T byl syntetizován firmou Sci-Tech (Praha, Česká Republika). BPA a jeho deuterovaný standard (d16-BPA), deuterovaný E2 (d3-E2) a kortizon (d7-kortizon), DHT, 2-hydrazinopyridin, ammonium formát, trifluorooctová kyselina, 99.9% tert-butyl methyl ether, aceton, hydrogenuhličitan sodný, hydroxid sodný a dansyl chlorid byly zakoupeny v Sigma-Aldrich (St. Louis, MO, USA). Metanol a voda pro chromatografii v HPLC kvalitě byly dodány firmou Merck (Darmstadt, Německo). Diethyl ether byl objednán ve firmě Lach-Ner, s.r.o. (Neratovice, Česká Republika).

5.4 Kapalinová chromatografie s tandemovou hmotnostní detekcí

Pro stanovení steroidů a BPA v plasmě a seminální plasmě byla použita kapalinová chromatografie Eksigent ultraLC 110 (Redwood City, CA, USA), která byla vybavena kolonou Kinetex C18 (100 x 3.0 mm, 2.6 µm, příp. 1.7 µm; Phenomenex, Torrance, CA, USA) a předkolonou Security Guard ULTRA cartridge system (UHPLC C18 pro 3mm průměr kolony; Phenomenex, Torrance, CA, USA).

Detekce analytů byla prováděna na hmotnostním detektoru API 3200 od firmy AB Sciex (AB Sciex, Concord, Kanada), kde probíhala ionizace elektrosprejem v pozitivním módu. Optimální podmínky iontového zdroje a hmotnostního detektoru byly zjištěny pomocí infuze 0.2 µg/mL jednotlivých analytů rychlostí 20 µL/min. Optimální

podmínky byly nalezeny při napětí iontového spreje 5500V, teplotě 600°C, hodnotě „curtain gas“ 25.0 psi, hodnotě kolizního plynu 4 psi, prvního plynu iontového zdroje 40.0 psi a hodnotě druhého plynu iontového zdroje 60.0 psi.

5.5 Další měření

LH, FSH a globulin vázající pohlavní hormony (SHBG) byly stanoveny imunoradiometrickou metodou formou kitu (Immunotech, Marseille, Francie). Plasmatický DHT byl stanoven radioimunoanalytickou metodou vyvinutou na našem pracovišti [113]. Koncentrace Se a Zn v plasmě byly stanoveny v laboratořích AGEL (Nový Jičín, ČR) atomovou absorpční spektrometrií, metodou akreditovanou a komerčně dostupnou. Hladiny 6 kongenerů PCB (PCB 28, 101, 118, 138, 153, 180) byly změřeny v akreditované laboratoři ALS Czech Republic (Pardubice, ČR) pomocí plynové chromatografie spojené s hmotnostní detekcí s vysokým rozlišením (GC-HRMS).

Hodnota volného testosteronu (FT) byla spočítána podle Vermeulena a kol. [114].

5.6 Statistická analýza

Na základě běžné praxe byla data pod limitem detekce (LOD) nahrazena hodnotou LOD/√2 [115]. Všechna data byla transformována kvůli významné šikmosti, špičatosti a nekonstatnímu rozptylu (heteroskedasticitě) pomocí Box-Coxovy transformace směrem k symetrii rozdělení data a reziduí a ke konstantnímu rozptylu (homoskedasticitě). Rozdíly mezi skupinami v jednotlivých analytech byly vyhodnoceny jednofaktorovou analýzou variance, která byla následována vícenásobným porovnáním metodou nejmenších významných rozdílů. Tyto statistické operace byly provedeny v softwaru Statgraphics Centurion XVI od firmy Statpoint Inc. (Warrenton, VA, USA). Vícerozměrná korelační analýza a s tím související vyhledání vícerozměrných nehomogenit byly provedena v software NCSS 2007 (Kaysville, UT, USA).

6 Výsledky

6.1 Vývin metody na stanovení steroidů a BPA v plasmě a seminální plasmě na UHPLC-MS/MS

Systém UHPLC-MS/MS, který máme v laboratoři, neumožnil stanovení všech steroidů bez větší přípravy kvůli malé citlivosti. Proto bylo nutné použít derivatizační činidlo, abychom zvýšili citlivost a rozlišení. Po vyzkoušení několika různých derivatizačních činidel byly vybrány 2 derivatizační činidla - 2-hydrazinopyridin pro stanovení steroidů s ketoskupinou a dansyl chlorid pro stanovení analytů s fenolovou skupinou - estrogenů (estron, 17β-estradiol, estriol) a BPA.

Obě metody byly optimalizovány jak z hlediska množství derivatizačního činidla, délky jeho působení a dalších kroků, jako je volba chromatografické kolony a nastavení vhodných podmínek pro hmotový spektrometr. Metoda na stanovení BPA a estrogenů byla navíc testována pro vyloučení možné kontaminace během pracovního postupu.

Druhá metoda byla vyvíjena ve 2 krocích. V první části byla metoda optimalizována pro stanovení 6 steroidů (Kortizol, kortizon, DHEA, 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA) v plasmě, vč. optimalizace volby extrakčního činidla a jeho objemu. Tato metoda byla následně rozšířena o stanovení další 4 steroidů (Preg, 17-OH-Preg, ADION, T) a validována jak pro plasmu, tak pro seminální plasmu.

Metody byly publikovány v časopisech s impakt faktorem, kde je detailněji popsán pracovní postup a výsledky validace ([112, 116] - Příloha VII, VIII a X).

6.2 Rozdíly v hladinách ED, steroidů a dalších relevantních analytů u 4 skupin mužů s různým stupněm neplodnosti

Průměrný věk (±SD) všech účastníků studie byl 35.8±5.6 let a průměrná hodnota BMI (±SD) byla 27.2±3.6 kg/m2. Do studie bylo zařazeno 55% mužů trpící nadváhou (BMI 25-30) a 20% obézních (BMI>30). Věk i hodnoty BMI se významně nelišily mezi 4 sledovanými skupinami mužů (Tab. 1).

BPA byl detekován v 89% plasmatických vzorcích a v 93% seminálních vzorcích. Mezi nejhojnější kongenery PCB se zařadili PCB 180 a PCB 153 (detekovány v 99 a

100% vzorků). Dále následovaly PCB 138 (v 96 % vzorků), PCB 118 (v 56 % vzorků), PCB 101 (v 8 % vzorků) a PCB 28 (5%).

Rozdíly v hladinách PCB mezi jednotlivými skupinami jsou uvedeny v Tab. 1. Hladiny seminálního BPA se zvyšovaly směrem k těžce neplodným mužům. Plasmatické hladiny BPA byly významně vyšší u mírně a středně neplodných mužů v porovnání se zdravými a těžce neplodnými muži. Součet hladin kongenerů byl nižší u mírně neplodných mužů v porovnání s ostatními skupinami. Pro samostatné kongenery PCB 101 a 28 nebylo vícenásobné porovnání provedeno z důvodu malého počtu vzorků s koncentracemi nad limitem detekce. Vícenásobné porovnání bylo provedeno stejným způsobem i pro steroidy v plasmě a seminální plasmě a další relevantní analyty v plasmě (LH, FSH, SHBG, Se, Zn) (Tab. 1 pro steroidy v seminální plasmě a ED a Tab. 2 pro steroidy v plasmě).

Tab. 1. Porovnání hladin vybraných ED (PCB v plasmě a BPA v plasmě a seminální plasmě) a steroidů v seminální plasmě u 4 skupin mužů s různým stupněm neplodnosti. Data jsou uvedena jako průměry s 95% konfidenčními intervaly (v závorkách) pro každou skupinu.
Skupina 1 = zdraví muži; Skupina 2 = mírně neplodní muži; Skupina 3= středně neplodní muži; Skupina 4 = těžce neplodní muži

	Analyt	Jednotky	**SKUPINA 1** (n=89)	**SKUPINA 2** (n=59)	**SKUPINA 3** (n=25)	**SKUPINA 4** (n=18)	Hladina významnosti p	Vícenásobné porovnání
	Věk	rok	35.9 (34.8; 37.0)	35.7 (34.3; 37.0)	35.8 (33.8; 37.8)	35.2 (32.9; 37.6)	0.972	
	BMI	kg/m²	27.7 (26.7;28.8)	26.9 (25.8;28.1)	26.1 (24.9; 27.5)	26.4 (24.8; 28.1)	0.275	
ED	Σ 6 PCB kongenerů	ng/g plasmy	1.52 (1.35;1.72)	1.30 (1.13;1.51)	1.00 (0.76;1.31)	1.32 (1.00;1.75)	**0.035**	3<1
	PCB 180	ng/g plasmy	0.66 (0.58;0.76)	0.53 (0.45;0.62]	0.49 (0.36;0.66)	0.57 (0.41;0.77)	0.098	2<1
	PCB 153	ng/g plasmy	0.57 (0.50;0.65)	0.48 (0.41;0.56)	0.35 (0.26;0.47)	0.47 (0.34;0.63)	**0.021**	3<1
	PCB 118	ng/g plasmy	0.030 (0.25;0.035)	0.026 (0.021;0.033)	0.025 (0.015;0.044)	0.020 (0.012;0.038)	0.552	
	PCB 138	ng/g plasmy	0.223 (0.195;0.256)	0.202 (0.173;0.237)	0.184 (0.137;0.252)	0.206 (0.147;0.295)	0.641	
	Plasmatický BPA	ng/mL	0.029 (0.019;0.044)	0.059(0.034;0.106)	0.072 (0.039;0.185)	0.019 (0.008;0.047)	**0.029**	1<2,3
	Seminální BPA	ng/mL	0.075 (0.055;0.100)	0.130 (0.093;0.179)	0.153 (0.091;0.243)	0.148 (0.082;0.250]	**0.018**	1<2,3,4
SEMINÁLNÍ PLASMA	Pregnenolon	ng/mL	0.198 (0.170;0.231]	0.173 (0.144;0.206)	0.298 (0.229;0.385)	0.182 (0.132;0.249)	**0.009**	1,2,4,<3
	17-OH-PREG	ng/mL	0.119 (0.090;0.154)	0.100 (0.072;0.136)	0.126 (0.080;0.186)	0.090 (0.048;0.151)	0.667	
	Kortizol	ng/mL	4.60 (3.74;5.55)	5.76 (4.67;6.97)	6.88 (5.15; 8.88)	4.88 (3.20;6.93)	0.105	1<3
	Kortizon	ng/mL	7.42 (6.78;8.06)	7.78 (7.05;8.51)	7.72 (6.64;8.81)	6.69 (5.41;7.99)	0.508	
	DHEA	ng/mL	1.40 (1.13;1.75)	1.45 (1.14;1.86)	2.40 (1.65;3.57)	1.88 (1.22;2.99)	0.073	1,2<3
	7α-OH-DHEA	ng/mL	0.275 (0.238;0.318)	0.235 (0.198;0.279)	0.245 (0.191;0.313)	0.223 (0.165;0.299)	0.429	
	7β-OH-DHEA	ng/mL	0.064 (0.053;0.075)	0.066 (0.054;0.079)	0.081 (0.061;0.103)	0.078 (0.056;0.103)	0.381	
	7-oxo-DHEA	ng/mL	0.035 (0.027;0.044)	0.039 (0.029;0.051)	0.040 (0.025;0.059)	0.039 (0.023;0.060]	0.917	
	Androstendion	ng/mL	0.016 (0.010;0.025)	0.022 (0.014;0.036)	0.016 (0.007;0.035)	0.011 (0.005;0.027)	0.528	
	Testosteron	ng/mL	0.019 (0.012;0.031)	0.020 (0.011;0.035)	0.023 (0.010;0.050)	0.018 (0.006;0.050)	0.975	
	DHT	ng/mL	0.225 (0.184;0.271)	0.158 (0.121;0.201)	0.135 (0.087;0.198)	0.114 (0.063;0.185)	**0.012**	1>2,3,4
	Estradiol	ng/mL	0.0039 (0.0034;0.0045)	0.0041 (0.0034;0.0050)	0.064 (0.0046;0.0097)	0.0050 (0.0036;0.0075)	**0.036**	1,2<3
	Estron	ng/mL	0.0043 (0.0037;0.0050)	0.0047(0.0039;0.0057)	0.0077 (0.0056;0.0109)	0.0047 (0.0034;0.0068)	**0.016**	1,2,4<3
	Estriol	ng/mL	0.043 (0.033;0.055)	0.031 (0.022;0.043)	0.048 (0.029;0.079)	0.065 (0.036;0.114)	0.128	

Tab. 2. Porovnání hladin steroidů v plasmě u 4 skupin mužů s různým stupněm neplodnosti. Data jsou uvedena jako průměry s 95% konfidenčními intervaly (v závorkách) pro každou skupinu.
Skupina 1 = zdraví muži; Skupina 2 = mírně neplodní muži; Skupina 3= středně neplodní muži; Skupina 4 = těžce neplodní muži

Analyt	Jednotky	**SKUPINA 1** (n=89)	**SKUPINA 2** (n=59)	**SKUPINA 3** (n=25)	**SKUPINA 4** (n=18)	Hladina významnosti p	Vícenásobné porovnání
Se	µmol/L	0.96 (0.91;1.01)	0.97 (0.92;1.04)	1.04 (0.93; 1.17)	1.03 (0.91;1.18)	0.512	
Zn	µmol/L	12.59 (11.80;13.44)	12.34 (11.43;13.33)	13.28 (11.60;15.22)	11.09 (9.55;12.90)	0.346	
LH	IU/L	2.80 (2.47;3.17)	3.40 (2.92;3.97)	3.25 (2.50;4.25)	5.39 (3.99;7.29)	**0.001**	1,2,3<4
FSH	IU/L	2.94 (2.57;3.37)	3.90 (3.29;4.66)	3.61(2.70;4.90)	10.24 (6.96;15.48)	**0.000**	1<2,4 2,3<4
SHBG	nmol/L	21.69 (19.65;23.87)	20.58 (18.24;23.14)	20.18 (16.34;24.64)	22.12 (17.42;27.70)	0.848	
Pregnenolon	ng/mL	0.36 (0.29;0.44)	0.33 (0.25;0.42)	0.47 (0.32; 0.66)	0.30 (0.17;0.47)	0.358	
17-OH-PREG	ng/mL	1.64 (1.41;1.90)	1.44 (1.21;1.73)	1.78 (1.34; 2.37)	1.26 (0.91; 1.77)	0.329	
Kortizol	ng/mL	106 (97;116)	106 (96;117)	97 (82;114)	87 (70;107)	0.266	
Kortizon	ng/mL	25.8 (24.5;27.2)	24.8 (23.3;26.4)	25.4 (23.0;27.9)	25.0 (22.2;27.9)	0.810	
DHEA	ng/mL	4.29 (3.81;4.85)	3.82 (3.33;4.41)	4.35 (3.49;5.48)	3.36 (2.62;4.36)	0.267	
7α-OH-DHEA	ng/mL	0.278 (0.244;0.317)	0.253 (0.217;0.295)	0.282 (0.222; 0.359)	0.209 (0.156;0.279)	0.285	
7β-OH-DHEA	ng/mL	0.149 (0.133;0.166)	0.126 (0.108;0.145)	0.139 (0.110;0.170)	0.104 (0.074;0.137)	0.064	1>4
7-oxo-DHEA	ng/mL	0.039 (0.032;0.048)	0.030 (0.023; 0.039)	0.034 (0.022;0.050)	0.042 (0.027;0.063)	0.336	
Androstendion	ng/mL	0.583 (0.532;0.638)	0.612 (0.549;0.683)	0.642 (0.536;0.769)	0.540 (0.436;0.670)	0.583	
Testosteron	ng/mL	3.32 (3.07;3.58)	3.34 (3.04;3.66)	3.75 (3.25;4.28)	3.37 (2.82;3.98)	0.494	
DHT	ng/mL	0.519 (0.497;0.541)	0.522 (0.486;0.549)	0.549 (0.504;0.597)	0.531 (0.480;0.585)	0.679	
Estradiol	ng/mL	0.017 (0.014;0.021)	0.016 (0.013;0.020)	0.016 (0.011; 0.022)	0.007 (0.004;0.012)	**0.016**	1,2,3>4
Estron	ng/mL	0.023 (0.019; 0.027)	0.022 (0.018;0.027)	0.022 (0.017; 0.029)	0.019 (0.013; 0.027)	0.854	
Estriol	ng/mL	0.009 (0.006;0.014)	0.006 (0.004;0.010)	0.006 (0.003;0.012)	0.010 (0.005;0.026)	0.474	

6.3 Vliv vybraných ED na parametry spermiogramu

Pomocí parciálních korelací adjustovaných na věk a BMI byly vyhodnoceny vztahy mezi jednotlivými ED a parametry spermiogramu – celkovým počtem spermií, koncentrací spermií, motilitou a morfologií (Tab. 3).

Tab. 3. Vliv vybraných ED na parametry spermiogramu

Parametry spermiogramu	Σ 6 PCB kongenerů v plasmě		BPA v plasmě		BPA v seminální plasmě	
	r	*p*-hodnota	*r*	*p*-hodnota	*r*	*p*-hodnota
Koncentrace (10^6/mL)	0.273	**0.000**	-0.119	0.134	-0.212	**0.005**
Celkový počet	0.308	**0.000**	-0.115	0.146	-0.178	**0.018**
Motilita	0.069	0.376	0.139	0.079	-0.106	0.164
- Progresivně pohyblivé spermie	0.112	0.147	0.095	0.233	-0.122	0.108
- Neprogresivně pohyblivé spermie	-0.064	0.411	0.192	**0.014**	-0.039	0.606
- Nepohyblivé spermie	-0.027	0.723	0.071	0.373	-0.037	0.626
Morfologie	0.099	0.223	-0.029	0.729	-0.156	**0.049**

Hladiny BPA v seminální tekutině negativně korelovaly s koncentrací, celkovým počtem spermií a morfologií, na druhou stranu tyto závislosti nebyly zjištěny u BPA v plasmě. Zde byla pozorována pozitivní korelace mezi BPA a počtem neprogresivně pohyblivých spermií. Hladiny PCB v plasmě překvapivě pozitivně korelovaly s koncentrací a celkovým počtem spermií.

6.4 Vliv vybraných ED na aktivitu 11βHSD a dalších enzymů

Pro zjištění aktivity některých enzymů steroidogeneze byly koncentrace steroidů dány do poměrů – vždy prekurzor ku produktu a hledány závislosti s vybranými ED. Vztahy jsou uvedeny jako parciální korelace adjustované na věk a BMI. Korelace podílů s plazmatickými ED byly vypočítány z plasmatických hladin, korelace se seminálním BPA byly spočítány z poměrů hladin steroidů v seminální plasmě (Tab. 4).

Významné parciální korelace byly nalezeny pro PCB a poměry Androstendion/T a T/DHT odpovídající enzymům 17βHSD a 5α-reduktáze. V periferii byl BPA asociován s poměrem kortizol/kortizon (enzym 11βHSD), DHEA/androstendion (enzym 3βHSD), androstendion/T (enzym 17βHSD či aldoketoreduktáza AKR1C3) a T/E2 (enzym aromatáza). Naopak v seminální plasmě nebyl nalezen žádný vztah mezi BPA a poměry steroidů, což naznačuje spíše

ovlivnění systémových enzymů steroidogeneze uložených v nadledvinách a periferních tkáních.

Tab. 4. Vliv PCB a BPA na aktivitu vybraných enzymů

Prekurzor/produkt	Σ 6 PCB kongenerů v plasmě		BPA v plasmě		BPA v seminální plasmě	
	r	*p*-hodnota	*r*	*p*-hodnota	*r*	*p*-hodnota
Kortizol/Kortizon	0.146	0.065	0.176	**0.026**	0.042	0.617
PREG/17-OH-PREG	-0.049	0.527	0.061	0.443	0.082	0.352
17-OH-Preg/DHEA	-0.030	0.703	0.151	0.058	-0.137	0.119
DHEA/Androstendion	0.103	0.190	0.168	**0.037**	-0.016	0.860
Androstendion/T	0.221	**0.004**	0.166	**0.037**	-0.118	0.220
T/DHT	-0.320	**0.000**	0.076	0.341	0.157	0.093
T/E2	-0.097	0.289	-0.372	**0.000**	0.037	0.695

6.5 Vliv vybraných ED na hladiny imunoaktivních a dalších steroidů

Pro hodnocení vztahů mezi imunoaktivními steroidy a ED byly opět vypočítány parciální korelace adjustované na věk a BMI (Tab. 5). Pro úplnost byly zjištěny i korelace s plasmatickými gonadotropiny a SHBG. Hladiny plasmatických ED byly korelovány s plasmatickými hladinami analytů a koncentrace BPA v seminální plasmě byla korelována s koncentracemi steroidů v seminální plasmě. Nebyl zde patrný vliv BPA ani PCB na hladiny plasmatických gonadotropinů a SHBG. Koncentrace 6 kongenerů PCB negativně korelovala s plasmatickými androgeny - T, DHT i volným T. Opačné výsledky byly pozorovány u BPA v plasmě a seminální plasmě u steroidů na začátku steroidní biosyntézy: BPA v plasmě pozitivně koreloval s PREG, 17-OH-PREG a DHEA v plasmě, naopak BPA v seminální plasmě s PREG a 17-OH-PREG koreloval negativně. Dále BPA v plasmě i seminální plasmě koreloval s plasmatickými, resp. seminálními estrogeny. Kromě pozitivní korelace mezi plasmatickým DHEA a BPA v plasmě nebyly zaznamenány žádné další významné vztahy mezi imunoaktivními steroidy.

Tab. 5. Vliv vybraných ED na hladiny imunoaktivních a dalších steroidů

Analyt	Σ 6 PCB kongenerů v plasmě		BPA v plasmě		BPA v seminální plasmě	
	r	*p*-hodnota	*r*	*p*-hodnota	*r*	*p*-hodnota
LH	-0.014	0.862	-0.021	0.792		
FSH	0.019	0.812	-0.111	0.166		
SHBG	-0.056	0.472	-0.011	0.893		
PREG	-0.021	0.786	0.239	**0.002**	-0.207	**0.011**
17-OH-PREG	0.022	0.778	0.264	**0.001**	-0.207	**0.015**
Kortizol	0.166	**0.035**	0.099	0.213	0.048	0.565
Kortizon	0.091	0.250	-0.077	0.331	0.049	0.553
DHEA	0.050	0.523	0.249	**0.001**	-0.125	0.129
7α-OH-DHEA	-0.128	0.103	0.069	0.386	0.082	0.329
7β-OH-DHEA	0.021	0.790	0.148	0.062	0.054	0.514
7-oxo-DHEA	-0.015	0.853	0.012	0.880	-0.109	0.210
Androstendion	-0.037	0.634	0.145	0.069	-0.037	0.681
T	-0.332	**0.000**	-0.044	0.577	0.105	0.266
FT	-0.337	**0.000**	-0.035	0.660		
DHT	-0.191	**0.013**	-0.177	**0.026**	-0.075	0.367
E2	-0.047	0.604	0.357	**0.000**	0.163	**0.033**
E1	0.138	0.131	0.286	**0.001**	0.061	0.423
E3	0.250	**0.006**	0.079	0.372	0.202	**0.009**

6.6 Vliv vybraných ED na hladiny stopových prvků

Parciální korelace adjustované na věk a BMI byly opět využity k odhalení vztahů mezi PCB a BPA a hladinami stopových prvků v plasmě (Tab. 6). Nebyly zde pozorovány žádné významné asociace.

Tab. 6. Vliv vybraných ED na hladiny Se, Zn

Analyty v plasmě	Σ 6 PCB kongenerů v plasmě		BPA v plasmě	
	r	*p*-hodnota	*r*	*p*-hodnota
Se	0.078	0.315	-0.001	0.989
Zn	-0.028	0.723	0.163	0.052

6.7 Korelace jednotlivých analytů v plasmě a seminální plasmě

Na závěr bylo zjištěno, nakolik jednotlivé analyty v plasmě korelují se svými protějšky v seminální plasmě (Tab. 7). Lze shrnout, že steroidy v obou biologických tekutinách spolu vzájemně korelují, kromě 7-oxo-DHEA, DHT, estronu a estradiolu.

Všechny tyto steroidy jsou syntetizovány zejména enzymy v periferii, naopak v testes se tvoří minoritně.

Tab. 7. Korelace jednotlivých steroidů a BPA ve 2 biologických tekutinách

Analyt	*r*	*p*-hodnota
BPA v plasmě vs. BPA v seminální plasmě	0.253	**0.001**
Kortizol v plasmě vs. kortizol v seminální plasmě	0.468	**0.000**
Kortizon v plasmě vs. kortizon v seminální plasmě	0.396	**0.000**
PREG v plasmě vs. PREG v seminální plasmě	0.375	**0.000**
17-OH-PREG v plasmě vs. 17-OH-PREG v seminální plasmě	0.351	**0.000**
DHEA v plasmě vs. DHEA v seminální plasmě	0.301	**0.000**
7α-OH-DHEA v plasmě vs. 7α-OH-DHEA v seminální plasmě	0.468	**0.000**
7β-OH-DHEA v plasmě vs. 7β-OH-DHEA v seminální plasmě	0.193	**0.000**
7-oxo-DHEA v plasmě vs. 7-oxo-DHEA v seminální plasmě	-0.041	0.645
Androstendion v plasmě vs. androstendion v seminální plasmě	0.192	**0.031**
T v plasmě vs. T v seminální plasmě	0.230	**0.013**
DHT v plasmě vs. DHT v seminální plasmě	-0.076	0.365
Estron v plasmě vs. estron v seminální plasmě	0.005	0.954
Estradiol v plasmě vs. estradiol v seminální plasmě	0.035	0.698
Estriol v plasmě vs. estriol v seminální plasmě	0.498	**0.000**

7 Diskuse

Působení ED na lidský organismus představuje v současnosti velice diskutovaný problém, jak na národní, tak na mezinárodní úrovni. Snahou Evropské Unie v současné době je stanovit kritéria pro posuzování výsledků testů ED, na jejichž základě budou jednotlivé ED na trhu omezeny, vyřazeny příp. ponechány.
Cílem práce bylo objasnit mechanismy působení ED na jednotlivé kroky lidské gonadální a adrenální steroidogeneze z hlediska mužské reprodukce. Uvedené informace by mohly přispět k prevenci negativního působení ED a pravděpodobně i k léčbě následků působení ED na mužský reprodukční systém.

Ve většině studií zkoumajících účinky ED na hladiny reprodukčních hormonů byly korelovány močové koncentrace ED s plasmatickými hladinami hormonů. Jen málo studií porovnávalo plasmatické hladiny ED s plasmatickými, příp. seminálními hladinami hormonů (shrnuto v článku [117], Příloha I). Vycházeli jsme z předpokladu, že hladiny ED měřené přímo v seminální tekutině budou nejlépe odrážet jejich působení na kvalitu spermií a steroidogenezi ve varlatech (shrnuto v článku [118],

Příloha II). Současné měření ED a steroidů v plasmě umožnilo zjistit, do jaké míry spolu korelují plasmatické a seminální koncentrace sledovaných analytů a odhadnout vliv ED na steroidogenezi v periferii.

V prvé řadě byla vyvinuta metoda na stanovení BPA a estrogenů (estron, estradiol, estriol) v plasmě a seminální plasmě ([112], Příloha VIII). Metodika stanovení BPA v plasmě/séru se již poměrně běžně používá [119-124]. Stanovení BPA v biologických tekutinách je v porovnání se stanoveními steroidů komplikovanější z důvodu možné kontaminace vzorků bisfenolem A během sběru, manipulace se vzorkem a jeho skladování. V každém kroku je nutno ověřit, zda nedochází k uvolňování zmiňované chemikálie z laboratorních plastů. V naší studii jsme sledovali možnou kontaminaci během celého procesu zacházení se vzorkem a dokázali jsme, že celková kontaminace BPA je pod limitem jeho detekce. Metoda tedy poskytuje citlivé a přesné stanovení nekonjugovaného BPA v plasmě i seminální plasmě.

Stanovení BPA v seminální plasmě již běžné není. Dosud byly publikovány 3 články na toto téma [120, 125, 126]. Inoue s kolektivem vyvinul instrumentální metodu na stanovení BPA ve spermatu bez využití derivatizace, proto jeho metoda měla poměrně vysoký limit detekce (LOD) – 100 pg/mL a limit kvantifikace (LOQ) 500 pg/mL [125]. Po porovnání se stanovením BPA pomocí enzymové imunoanalýzy na pevné fázi (ELISA) autoři shrnuli, že výsledky z ELISA metody mohou poskytovat nepřesné výsledky kvůli matricovým efektům a nedostatečné specifitě protilátky proti BPA [120, 125, 127]. V další zmiňované studii autoři měřili BPA v seminální plasmě, překvapivě však všechny jejich měřené vzorky byly pod LOD, který činil 1 pg/mL [126]. Další studie měřila BPA radioimunoanalyticky v seminální tekutině a folikulární tekutině u 28 náhodně vybraných párů podstupující *in vitro* fertilizaci. Koncentrace BPA v seminální tekutině se pohybovaly od 80 pg/mL do 1 ng/mL [120]. Naše metoda s limitem kvantifikace 28.9 pg/mL pro seminální plasmu a 43.5 pg/mL pro plasmu umožnila stanovení BPA v 93% vzorků seminální plasmy a 89% vzorků plasmy. Koncentrace estriolu v seminální plasmě byly v tomto článku publikovány poprvé.

Druhá metoda na stanovení steroidů v plasmě a seminální plasmě umožnila stanovit v plasmě 10 steroidů a v seminální plasmě 11 steroidů. Steroidem stanovovaným navíc v seminální plasmě oproti krevní plasmě byl DHT, který v plasmě nedosáhl uspokojivých validačních výsledků. V porovnání s dřívějšími

imunoanalytickými metodami vyvinutými na našem pracovišti i dalších pracovištích tato metoda umožní rychlejší, přesnější a citlivější stanovení za použití 500 µL krevní plasmy nebo 1 mL seminální plasmy pro celou analýzu. Stanovení komplexního spektra steroidů umožnilo sledovat vliv ED na celou základní steroidogenezi.

V naší studii BPA v plasmě jen mírně koreloval s BPA v seminální plasmě a jen BPA v seminální plasmě negativně ovlivňoval koncentraci spermií, jejich morfologii a celkový počet spermií. Těchto výsledků jsme dosáhli jak na menším počtu pacientů, tak poté na celé skupině 191 mužů ([128] – Příloha IX a Příloha X). Podobné výsledky byly publikovány v několika studiích [129-131], na druhou stranu jiné studie tyto vztahy neprokázaly [132-134]. Všechny tyto studie měřily BPA v moči a ani v rámci této matrice nebylo dosaženo shody ve výsledcích. Stanovení močových analytů reflektuje spíše jejich rychlost vylučování, plasmatické hladiny nekonjugovaného BPA již dokumentují více jejich biologickou dostupnost pro tkáně. Seminální plasma se zdá být optimální pro studium vztahů mezi kvalitou spermií a vlivem ED, protože se nachází nejblíže zrání spermií, ovšem jsou zde jiné omezení, jako je její horší dostupnost a malý objem vzorku. Dále výběr populace může vést k odlišným závěrům, kde v některých studiích byla zkoumána obecná populace [132, 133], v dalších plodní muži [134], muži navštěvující centra asistované reprodukce (naše studie, [129, 131]) či muži vystaveni velké expozici ED [130].

Vztahy mezi vlivem PCB a kvalitou spermií byly shrnuty v přehledovém článku od Meekera a Hausera [45]. Autoři ukázali konzistentní vztah mezi PCB a zhoršenou motilitou, který v naší studii potvrzen nebyl. Dále bylo zjištěno, že hladiny PCB v plasmě byli nižší u zdravých mužů v porovnání se středně neplodnými muži (Tab. 1) a současně pozitivně korelovaly s celkovým počtem spermií a jejich koncentrací. Podobné výsledky byly uvedeny ve studii Dallingy a kol. [47], kde byly zjištěny vyšší hladiny PCB u mužů s dobrou kvalitou spermií v porovnání s muži s horší kvalitou spermií. Tyto výsledky byly na hranici významnosti ($p=0.06$). Podobně jako studie výše uvedených autorů i naše práce naznačuje, že horší kvalita spermií není zapříčiněna expozicí PCB, ale jinými, dosud neznámými faktory.

Na téma vlivu močového BPA na hladiny plasmatických reprodukčních hormonů bylo publikováno více studií, ovšem s protichůdnými výsledky [133-140]. Naše zjištění o pozitivní korelaci mezi BPA v obou tělních tekutinách s estrogeny bylo v souladu se třemi z nich [133, 136, 137]. Jedno z možných vysvětlení zvýšených hladin estrogenů a sníženého poměru T/E2 v plasmě by mohlo být zvýšení aktivity či

exprese aromatázy na periferii nebo přímo ve varlatech. Vysvětlením by mohlo být také pomalejší odbourávání estrogenů pomocí sulfotransferáz či UDP-glukuronosyltransferáz. Podle jedné studie je i BPA glukuronidován při katalýze různými izoformami UDP-glukuronosyltransferáz [141]. Mohlo by tedy jít o kompetici na těchto enzymech. *In vitro* studie od Zhanga a kol. na H295R buňkách podporuje tuto hypotézu [142]. Další studie nepotvrdili vztah mezi BPA a E2 [134, 135].

Protichůdné výsledky byly publikovány také ohledně vztahu BPA a plasmatických gonadotropinů. V této studii BPA neměl statisticky významný vliv ani na jeden z gonadotropinů, stejně jako ve studii Mendioly a kol.[134]. Studie autorů Lassen a kol. uvádí vyšší hladiny LH vlivem BPA, bez změny koncentrací FSH [133]. Na druhou stranu Meeker a kol. nalézají vyšší hladiny FSH, ale ne LH ve vztahu k BPA [138]. Další studie na zvířecích modelech naopak prokazovaly potlačení tvorby obou gonadotropinu po expozici BPA [98, 143], zatímco jiná studie publikovala zvýšení produkce LH [144]. Z takto nekonzistentních výsledků je samozřejmě obtížné vyvodit jednoznačné závěry. Pro vyhodnocení vztahu mezi gonadotropiny a BPA bude tedy zapotřebí uskutečnit více studií.

Naše výsledky ohledně snížení plasmatické koncentrace T ve vztahu k PCB jsou v souladu s některými studiemi jiných autorů [50, 51]. Stejné výsledky byly získány u adolescentů, kteří byli působení PCB vystaveni prenatálně [145]. Další studie publikovaly negativní korelace PCB s T vázaným na SHBG [52, 53] či FT [48, 54], zatímco jiné studie nenalezly žádný vztah mezi PCB a T [146, 147].

Pokud je nám známo toto je první studie zabývající se vlivem ED na hlavní kroky steroidogeneze (Tab. 5). Zatímco v krevní plasmě prekurzory pohlavních hormonů (PREG, 17-OH-PREG a DHEA) pozitivně korelovaly s BPA, situace v seminální plasmě byla odlišná. BPA s PREG a 17-OH-PREG zde koreloval negativně. Plasmatické koncentrace Δ^5 steroidů odrážejí hlavně jejich produkci v nadledvinách [148], zatímco v seminální plasmě předpokládáme převážně testikulární původ Δ^5 steroidů [117] (Příloha I). Vzhledem k tomu že na poměry PREG/17-OH-PREG v plasmě i seminální plasmě BPA vliv nemá, snížení produkce prekurzorů reprodukčních hormonů ve varlatech by mohlo být způsobeno sníženou dodávkou substrátu do mitochondrií (snížení aktivity či exprese cholesterol desmolázy či StAR). Dřívější *in vitro* studie a studie na myších prokázaly snížení exprese StAR proteinu po expozici BPA [149, 150] ve shodě s naší hypotézou. V plasmě byla situace obrácená, možným vysvětlením je naopak zvýšená dodávka

cholesterolu do mitochondrie nebo snížení aktivity či exprese enzymů stojící dále ve steroidní cestě a tím akumulace prekurzorů.

Podíl kortizol/kortizon neodráží jen aktivitu nadledvin, ale také lokální interkonverzi mezi kortizolem a kortizonem v jednotlivých tkáních katalyzovanou 11βHSD (typ 1 nebo 2) [151] (Příloha VI). PCB neměly vliv na podíl kortizol/kortizon a BPA mírně koreloval s tímto podílem v plasmě, ale už ne v seminální plasmě, kde jsme tento vztah předpokládali. Korelace obou ED v plasmě s podílem ADION/T naznačuje snížení aktivity systémových (periferních) 17β-hydroxysteroidních dehydrogenáz. Zajímavá je i negativní korelace plasmatických PCB s poměrem T/DHT ukazující jejich negativní vliv na systémové hladiny T. Kromě potlačení syntézy na periferii může tento výsledek znamenat zvýšení aktivity 5α-reduktázy. V neposlední řadě byla zaznamenána negativní asociace plasmatického BPA s T/E2 a vyšší hladiny jak plasmatických, tak seminálních hladin estrogenů, které byly již diskutovány výše. Všechny tyto výsledky ukazují na různé mechanismy účinku studovaných ED.

Poprvé byly stanoveny koncentrace ED společně s hladinami 7-hydroxylovaných metabolitů DHEA, o kterých se předpokládají imunomodulační a imunoprotektivní účinky [152-154]. Tyto metabolity jsou přítomné i v seminální plasmě [155], kde by mohly působit proti nepříznivým účinkům glukokortikoidů na testikulární steroidogenezi. Ačkoli jsme pozorovali zvýšené hladiny plasmatické DHEA ve vztahu k BPA, žádné další korelace mezi 7-hydroxylovanými metabolity DHEA a ED nebyly nalezeny ani v plasmě ani v seminální plasmě.

Nebyl nalezen ani žádný vztah mezi hladinami Se a Zn v plasmě a kvalitou spermií, ačkoli se obecně uznává, že tyto stopové prvky mají příznivý vliv na spermatogenezi [156-158].

8 Závěry

- Dvě metody na stanovení BPA a 10 steroidů v plasmě či 11 steroidů v seminální plasmě byly vyvinuty a validovány. Obě metody poskytují nízké limity kvantifikace, přesnost a selektivitu.
- Environmentální hladiny PCB nemají vliv na kvalitu spermií u studované populace mužů z ČR.

- Byla však prokázána negativní korelace mezi PCB a hladinami T, FT a DHT v krvi.

- Zvýšené hladiny BPA v seminální plasmě byly spojené se signifikantně sníženým celkovým počtem spermií, koncentrací a morfologií spermií. BPA tedy představuje nepříznivý faktor pro lidskou reprodukci.

- Výsledky dále naznačují, že BPA snižuje rychlost steroidogeneze v testes v Δ^5 cestě, naopak tato cesta byla stimulována v periferii, tedy v nadledvinách. Další narušení steroidogeneze se zřejmě odehrává na úrovni estrogenního metabolismu, kdy dochází buď k potlačení estrogenního katabolismu či zvýšení aktivity či exprese aromatázy.

9 Shrnutí závěrů práce

Byly vyvinuty 2 metody na stanovení BPA a 10 steroidů v plasmě a 11 steroidů v seminální plasmě, které umožnily stanovení u 191 mužů. BPA představuje nepříznivý faktor pro lidskou reprodukci snižováním kvality spermií a ovlivněním hladin steroidů. PCB působí negativně na koncentrace androgenů v krvi. Výsledky nepotvrdily původní hypotézu o ovlivnění aktivity 11βHSD endokrinními disruptory, ukázaly však na jiný mechanismus účinku ED v organismu.

Disertační práce byla podpořena projektem Interní grantové agentury Ministerstva zdravotnictví NT/13369.

10 Literatura

1. E. Diamanti-Kandarakis, J.P. Bourguignon, L.C. Giudice, R. Hauser, G.S. Prins, A.M. Soto, R.T. Zoeller, and A.C. Gore, Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev. 30(4) (2009): p. 293-342.
2. U.U.N.E.P.a.W.W.H. Organization). State of the science of endocrine disrupting chemicals - 2012. 2012 2.11.2015].
3. E. Carlsen, A. Giwercman, N. Keiding, and N.E. Skakkebaek, Evidence for decreasing quality of semen during past 50 years. BMJ. 305(6854) (1992): p. 609-13.
4. J. Auger, J.M. Kunstmann, F. Czyglik, and P. Jouannet, Decline in semen quality among fertile men in Paris during the past 20 years. N Engl J Med. 332(5) (1995): p. 281-5.

5. S. Irvine, E. Cawood, D. Richardson, E. MacDonald, and J. Aitken, Evidence of deteriorating semen quality in the United Kingdom: birth cohort study in 577 men in Scotland over 11 years. BMJ. 312(7029) (1996): p. 467-71.
6. S.H. Swan, E.P. Elkin, and L. Fenster, Have sperm densities declined? A reanalysis of global trend data. Environ Health Perspect. 105(11) (1997): p. 1228-32.
7. H. Fisch, E.T. Goluboff, J.H. Olson, J. Feldshuh, S.J. Broder, and D.H. Barad, Semen analyses in 1,283 men from the United States over a 25-year period: no decline in quality. Fertil Steril. 65(5) (1996): p. 1009-14.
8. J.T. Seo, K.H. Rha, Y.S. Park, and M.S. Lee, Semen quality over a 10-year period in 22,249 men in Korea. Int J Androl. 23(4) (2000): p. 194-8.
9. A.M. Andersson, T.K. Jensen, A. Juul, J.H. Petersen, T. Jorgensen, and N.E. Skakkebaek, Secular decline in male testosterone and sex hormone binding globulin serum levels in Danish population surveys. J Clin Endocrinol Metab. 92(12) (2007): p. 4696-705.
10. H.A. Feldman, C. Longcope, C.A. Derby, C.B. Johannes, A.B. Araujo, A.D. Coviello, W.J. Bremner, and J.B. McKinlay, Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts male aging study. J Clin Endocrinol Metab. 87(2) (2002): p. 589-98.
11. H.O. Adami, R. Bergstrom, M. Mohner, W. Zatonski, H. Storm, A. Ekbom, S. Tretli, L. Teppo, H. Ziegler, M. Rahu, and et al., Testicular cancer in nine northern European countries. Int J Cancer. 59(1) (1994): p. 33-8.
12. C. Chilvers, M.C. Pike, D. Forman, K. Fogelman, and M.E. Wadsworth, Apparent doubling of frequency of undescended testis in England and Wales in 1962-81. Lancet. 2(8398) (1984): p. 330-2.
13. L.J. Paulozzi, International trends in rates of hypospadias and cryptorchidism. Environ Health Perspect. 107(4) (1999): p. 297-302.
14. P.D. Darbre, *Entry into human tissues*, in *Endocrine disruption and human health*. 2015, Academic Press.
15. L. Trasande, R.T. Zoeller, U. Hass, A. Kortenkamp, P. Grandjean, J.P. Myers, J. DiGangi, M. Bellanger, R. Hauser, J. Legler, N.E. Skakkebaek, and J.J. Heindel, Estimating burden and disease costs of exposure to endocrine-disrupting chemicals in the European union. J Clin Endocrinol Metab. 100(4) (2015): p. 1245-55.
16. R. Hauser, N.E. Skakkebaek, U. Hass, J. Toppari, A. Juul, A.M. Andersson, A. Kortenkamp, J.J. Heindel, and L. Trasande, Male reproductive disorders, diseases, and costs of exposure to endocrine-disrupting chemicals in the European Union. J Clin Endocrinol Metab. 100(4) (2015): p. 1267-77.
17. G. Bleau, J. Lemarbre, G. Faucher, K.D. Roberts, and A. Chapdelaine, Semen selenium and human fertility. Fertil Steril. 42(6) (1984): p. 890-4.
18. R. Scott, A. MacPherson, R.W. Yates, B. Hussain, and J. Dixon, The effect of oral selenium supplementation on human sperm motility. Br J Urol. 82(1) (1998): p. 76-80.
19. A.H. Colagar, E.T. Marzony, and M.J. Chaichi, Zinc levels in seminal plasma are associated with sperm quality in fertile and infertile men. Nutr Res. 29(2) (2009): p. 82-8.
20. S.E. Chia, C.N. Ong, L.H. Chua, L.M. Ho, and S.K. Tay, Comparison of zinc concentrations in blood and seminal plasma and the various sperm parameters between fertile and infertile men. J Androl. 21(1) (2000): p. 53-7.
21. D.J. Barker, The developmental origins of adult disease. Eur J Epidemiol. 18(8) (2003): p. 733-6.
22. S.F. Arnold, D.M. Klotz, B.M. Collins, P.M. Vonier, L.J. Guillette, Jr., and J.A. McLachlan, Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science. 272(5267) (1996): p. 1489-92.
23. H. Sharma, P. Zhang, D.S. Barber, and B. Liu, Organochlorine pesticides dieldrin and lindane induce cooperative toxicity in dopaminergic neurons: role of oxidative stress. Neurotoxicology. 31(2) (2010): p. 215-22.

24. M.G. Wade, D. Desaulniers, K. Leingartner, and W.G. Foster, Interactions between endosulfan and dieldrin on estrogen-mediated processes in vitro and in vivo. Reprod Toxicol. 11(6) (1997): p. 791-8.
25. J.D. Meeker, Exposure to environmental endocrine disrupting compounds and men's health. Maturitas. 66(3) (2010): p. 236-41.
26. R. Hauser, J.S. Barthold, and J.D. Meeker, *Epidemiologic evidence on the relationship between environmental endocrine disruptors and male reproductive and developmental health*, in *Endocrine-Disrupting Chemicals: From Basic Research to Clinical Practice*, A.C. Gore, Editor. 2010, Humana Press: Totowa, NJ.
27. W.V. Welshons, S.C. Nagel, and F.S. vom Saal, Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology. 147(6 Suppl) (2006): p. S56-69.
28. B.S. Rubin, Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. J Steroid Biochem Mol Biol. 127(1-2) (2011): p. 27-34.
29. J.A. Brotons, M.F. Olea-Serrano, M. Villalobos, V. Pedraza, and N. Olea, Xenoestrogens released from lacquer coatings in food cans. Environ Health Perspect. 103(6) (1995): p. 608-12.
30. A.V. Krishnan, P. Stathis, S.F. Permuth, L. Tokes, and D. Feldman, Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology. 132(6) (1993): p. 2279-86.
31. J.H. Kang, K. Kito, and F. Kondo, Factors influencing the migration of bisphenol A from cans. J Food Prot. 66(8) (2003): p. 1444-7.
32. E.J. Routledge, R. White, M.G. Parker, and J.P. Sumpter, Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and ERbeta. J Biol Chem. 275(46) (2000): p. 35986-93.
33. J.C. Gould, L.S. Leonard, S.C. Maness, B.L. Wagner, K. Conner, T. Zacharewski, S. Safe, D.P. McDonnell, and K.W. Gaido, Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. Mol Cell Endocrinol. 142(1-2) (1998): p. 203-14.
34. G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, and J.A. Gustafsson, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology. 139(10) (1998): p. 4252-63.
35. H. Okada, T. Tokunaga, X. Liu, S. Takayanagi, A. Matsushima, and Y. Shimohigashi, Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor-gamma. Environ Health Perspect. 116(1) (2008): p. 32-8.
36. V. Delfosse, M. Grimaldi, A. le Maire, W. Bourguet, and P. Balaguer, Nuclear receptor profiling of bisphenol-A and its halogenated analogues. Vitam Horm. 94(2014): p. 229-51.
37. H.J. Lee, S. Chattopadhyay, E.Y. Gong, R.S. Ahn, and K. Lee, Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. Toxicol Sci. 75(1) (2003): p. 40-6.
38. C. Teng, B. Goodwin, K. Shockley, M. Xia, R. Huang, J. Norris, B.A. Merrick, A.M. Jetten, C.P. Austin, and R.R. Tice, Bisphenol A affects androgen receptor function via multiple mechanisms. Chem Biol Interact. 203(3) (2013): p. 556-64.
39. K. Moriyama, T. Tagami, T. Akamizu, T. Usui, M. Saijo, N. Kanamoto, Y. Hataya, A. Shimatsu, H. Kuzuya, and K. Nakao, Thyroid hormone action is disrupted by bisphenol A as an antagonist. J Clin Endocrinol Metab. 87(11) (2002): p. 5185-90.
40. R.M. Sargis, D.N. Johnson, R.A. Choudhury, and M.J. Brady, Environmental endocrine disruptors promote adipogenesis in the 3T3-L1 cell line through glucocorticoid receptor activation. Obesity (Silver Spring). 18(7) (2010): p. 1283-8.
41. A. Pereira-Fernandes, H. Demaegdt, K. Vandermeiren, T.L. Hectors, P.G. Jorens, R. Blust, and C. Vanparys, Evaluation of a screening system for obesogenic compounds: screening of endocrine disrupting compounds and evaluation of the PPAR dependency of the effect. PLoS One. 8(10) (2013): p. e77481.

42. Y.F. Wang, H.R. Chao, C.H. Wu, C.H. Tseng, Y.T. Kuo, and T.C. Tsou, A recombinant peroxisome proliferator response element-driven luciferase assay for evaluation of potential environmental obesogens. Biotechnol Lett. 32(12) (2010): p. 1789-96.
43. Y. Sui, N. Ai, S.H. Park, J. Rios-Pilier, J.T. Perkins, W.J. Welsh, and C. Zhou, Bisphenol A and its analogues activate human pregnane X receptor. Environ Health Perspect. 120(3) (2012): p. 399-405.
44. P. Alonso-Magdalena, O. Laribi, A.B. Ropero, E. Fuentes, C. Ripoll, B. Soria, and A. Nadal, Low doses of bisphenol A and diethylstilbestrol impair Ca2+ signals in pancreatic alpha-cells through a nonclassical membrane estrogen receptor within intact islets of Langerhans. Environ Health Perspect. 113(8) (2005): p. 969-77.
45. J.D. Meeker and R. Hauser, Exposure to polychlorinated biphenyls (PCBs) and male reproduction. Syst Biol Reprod Med. 56(2) (2010): p. 122-31.
46. R. Rozati, P.P. Reddy, P. Reddanna, and R. Mujtaba, Role of environmental estrogens in the deterioration of male factor fertility. Fertil Steril. 78(6) (2002): p. 1187-94.
47. J.W. Dallinga, E.J. Moonen, J.C. Dumoulin, J.L. Evers, J.P. Geraedts, and J.C. Kleinjans, Decreased human semen quality and organochlorine compounds in blood. Hum Reprod. 17(8) (2002): p. 1973-9.
48. J. Richthoff, L. Rylander, B.A. Jonsson, H. Akesson, L. Hagmar, P. Nilsson-Ehle, M. Stridsberg, and A. Giwercman, Serum levels of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) in relation to markers of reproductive function in young males from the general Swedish population. Environ Health Perspect. 111(4) (2003): p. 409-13.
49. R. Hauser, Z. Chen, L. Pothier, L. Ryan, and L. Altshul, The relationship between human semen parameters and environmental exposure to polychlorinated biphenyls and p,p'-DDE. Environ Health Perspect. 111(12) (2003): p. 1505-11.
50. A. Goncharov, R. Rej, S. Negoita, M. Schymura, A. Santiago-Rivera, G. Morse, and D.O. Carpenter, Lower serum testosterone associated with elevated polychlorinated biphenyl concentrations in Native American men. Environ Health Perspect. 117(9) (2009): p. 1454-60.
51. L.M. Schell, M.V. Gallo, G.D. Deane, K.R. Nelder, A.P. DeCaprio, and A. Jacobs, Relationships of polychlorinated biphenyls and dichlorodiphenyldichloroethylene (p,p'-DDE) with testosterone levels in adolescent males. Environ Health Perspect. 122(3) (2014): p. 304-9.
52. V. Persky, M. Turyk, H.A. Anderson, L.P. Hanrahan, C. Falk, D.N. Steenport, R. Chatterton, Jr., and S. Freels, The effects of PCB exposure and fish consumption on endogenous hormones. Environ Health Perspect. 109(12) (2001): p. 1275-83.
53. M.E. Turyk, H.A. Anderson, S. Freels, R. Chatterton, Jr., L.L. Needham, D.G. Patterson, Jr., D.N. Steenport, L. Knobeloch, P. Imm, and V.W. Persky, Associations of organochlorines with endogenous hormones in male Great Lakes fish consumers and nonconsumers. Environ Res. 102(3) (2006): p. 299-307.
54. J.P. Bonde, G. Toft, L. Rylander, A. Rignell-Hydbom, A. Giwercman, M. Spano, G.C. Manicardi, D. Bizzaro, J.K. Ludwicki, V. Zvyezday, E.C. Bonefeld-Jorgensen, H.S. Pedersen, B.A. Jonsson, and A.M. Thulstrup, Fertility and markers of male reproductive function in Inuit and European populations spanning large contrasts in blood levels of persistent organochlorines. Environ Health Perspect. 116(3) (2008): p. 269-77.
55. K.P. Phillips and N. Tanphaichitr, Human exposure to endocrine disrupters and semen quality. J Toxicol Environ Health B Crit Rev. 11(3-4) (2008): p. 188-220.
56. D.G. de Rooij and L.D. Russell, All you wanted to know about spermatogonia but were afraid to ask. J Androl. 21(6) (2000): p. 776-98.
57. L. O'Donnell, K.M. Robertson, M.E. Jones, and E.R. Simpson, Estrogen and spermatogenesis. Endocr Rev. 22(3) (2001): p. 289-318.
58. W.H. Walker and J. Cheng, FSH and testosterone signaling in Sertoli cells. Reproduction. 130(1) (2005): p. 15-28.
59. M.D. Griswold, The central role of Sertoli cells in spermatogenesis. Semin Cell Dev Biol. 9(4) (1998): p. 411-6.

60. R.S. Wang, S. Yeh, C.R. Tzeng, and C. Chang, Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocr Rev. 30(2) (2009): p. 119-32.
61. R. Lagos-Cabre and R.D. Moreno, Contribution of environmental pollutants to male infertily: a working model of germ cell apoptosis induced by plasticizers. Biol Res. 45(1) (2012): p. 5-14.
62. R.M. Sharpe, Environmental/lifestyle effects on spermatogenesis. Philos Trans R Soc Lond B Biol Sci. 365(1546) (2010): p. 1697-712.
63. M.L. Dufau, The luteinizing hormone receptor. Annu Rev Physiol. 60(1998): p. 461-96.
64. F. Arakane, S.R. King, Y. Du, C.B. Kallen, L.P. Walsh, H. Watari, D.M. Stocco, and J.F. Strauss, 3rd, Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. J Biol Chem. 272(51) (1997): p. 32656-62.
65. V. Papadopoulos, In search of the function of the peripheral-type benzodiazepine receptor. Endocr Res. 30(4) (2004): p. 677-84.
66. D.M. Stocco, StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol. 63(2001): p. 193-213.
67. T. Hauet, J. Liu, H. Li, M. Gazouli, M. Culty, and V. Papadopoulos, PBR, StAR, and PKA: partners in cholesterol transport in steroidogenic cells. Endocr Res. 28(4) (2002): p. 395-401.
68. L.T. Van, Assessment of steroidogenesis and steroidogenic enzyme functions. Journal of Steroid Biochemistry and Molecular Biology. 137(2013): p. 176-182.
69. L.F. Silveira, M.G. Teles, E.B. Trarbach, and A.C. Latronico, Role of kisspeptin/GPR54 system in human reproductive axis. Front Horm Res. 39(2010): p. 13-24.
70. A.K. Roseweir and R.P. Millar, The role of kisspeptin in the control of gonadotrophin secretion. Hum Reprod Update. 15(2) (2009): p. 203-12.
71. N.E. Rance, Menopause and the human hypothalamus: evidence for the role of kisspeptin/neurokinin B neurons in the regulation of estrogen negative feedback. Peptides. 30(1) (2009): p. 111-22.
72. J.T. Smith, M.J. Cunningham, E.F. Rissman, D.K. Clifton, and R.A. Steiner, Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology. 146(9) (2005): p. 3686-92.
73. N. de Roux, E. Genin, J.C. Carel, F. Matsuda, J.L. Chaussain, and E. Milgrom, Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci U S A. 100(19) (2003): p. 10972-6.
74. S.B. Seminara, S. Messager, E.E. Chatzidaki, R.R. Thresher, J.S. Acierno, Jr., J.K. Shagoury, Y. Bo-Abbas, W. Kuohung, K.M. Schwinof, A.G. Hendrick, D. Zahn, J. Dixon, U.B. Kaiser, S.A. Slaugenhaupt, J.F. Gusella, S. O'Rahilly, M.B. Carlton, W.F. Crowley, Jr., S.A. Aparicio, and W.H. Colledge, The GPR54 gene as a regulator of puberty. N Engl J Med. 349(17) (2003): p. 1614-27.
75. P.J. Illingworth, N.P. Groome, W. Byrd, W.E. Rainey, A.S. McNeilly, J.P. Mather, and W.J. Bremner, Inhibin-B: a likely candidate for the physiologically important form of inhibin in men. J Clin Endocrinol Metab. 81(4) (1996): p. 1321-5.
76. B.D. Anawalt, A.M. Bebb Ra Fau - Matsumoto, N.P. Matsumoto Am Fau - Groome, P.J. Groome Np Fau - Illingworth, A.S. Illingworth Pj Fau - McNeilly, W.J. McNeilly As Fau - Bremner, and W.J. Bremner, Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. (0021-972X (Print)).
77. R.A. Anderson, C. Irvine Ds Fau - Balfour, N.P. Balfour C Fau - Groome, S.C. Groome Np Fau - Riley, and S.C. Riley, Inhibin B in seminal plasma: testicular origin and relationship to spermatogenesis. (0268-1161 (Print)).
78. P.K. Nicholls, P.G. Stanton, J.L. Chen, J.S. Olcorn, J.T. Haverfield, H. Qian, K.L. Walton, P. Gregorevic, and C.A. Harrison, Activin signaling regulates Sertoli cell differentiation and function. Endocrinology. 153(12) (2012): p. 6065-77.

79. P. Kumanov, A. Nandipati Kc Fau - Tomova, R. Tomova A Fau - Robeva, A. Robeva R Fau - Agarwal, and A. Agarwal, Significance of inhibin in reproductive pathophysiology and current clinical applications. (1472-6483 (Print)).
80. A.M. Trbovich, F.H. Martinelle N Fau - O'Neill, E.J. O'Neill Fh Fau - Pearson, P.K. Pearson Ej Fau - Donahoe, P.M. Donahoe Pk Fau - Sluss, J. Sluss Pm Fau - Teixeira, and J. Teixeira, Steroidogenic activities in MA-10 Leydig cells are differentially altered by cAMP and Mullerian inhibiting substance. (0960-0760 (Print)).
81. W.R. Kelce, L.E. Gray, and E.M. Wilson, Antiandrogens as environmental endocrine disruptors. Reprod Fertil Dev. 10(1) (1998): p. 105-11.
82. J.C. Davey, A.P. Nomikos, M. Wungjiranirun, J.R. Sherman, L. Ingram, C. Batki, J.P. Lariviere, and J.W. Hamilton, Arsenic as an endocrine disruptor: arsenic disrupts retinoic acid receptor- and thyroid hormone receptor-mediated gene regulation and thyroid hormone-mediated amphibian tail metamorphosis. Environ Health Perspect. 116(2) (2008): p. 165-72.
83. M.L. Scippo, C. Argiris, C. Van De Weerdt, M. Muller, P. Willemsen, J. Martial, and G. Maghuin-Rogister, Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. Anal Bioanal Chem. 378(3) (2004): p. 664-9.
84. M.K. Sarath Josh, S. Pradeep, K.S. Vijayalekshmi Amma, S. Balachandran, U.C. Abdul Jaleel, M. Doble, F. Spener, and S. Benjamin, Phthalates efficiently bind to human peroxisome proliferator activated receptor and retinoid X receptor alpha, beta, gamma subtypes: an in silico approach. J Appl Toxicol,(2013).
85. A. Ziv-Gal, Z.R. Craig, W. Wang, and J.A. Flaws, Bisphenol A inhibits cultured mouse ovarian follicle growth partially via the aryl hydrocarbon receptor signaling pathway. Reprod Toxicol. 42C(2013): p. 58-67.
86. J. Mimura and Y. Fujii-Kuriyama, Functional role of AhR in the expression of toxic effects by TCDD. Biochim Biophys Acta. 1619(3) (2003): p. 263-8.
87. J.N. Feige, L. Gelman, D. Rossi, V. Zoete, R. Metivier, C. Tudor, S.I. Anghel, A. Grosdidier, C. Lathion, Y. Engelborghs, O. Michielin, W. Wahli, and B. Desvergne, The endocrine disruptor monoethyl-hexyl-phthalate is a selective peroxisome proliferator-activated receptor gamma modulator that promotes adipogenesis. J Biol Chem. 282(26) (2007): p. 19152-66.
88. J. Kwintkiewicz, Y. Nishi, T. Yanase, and L.C. Giudice, Peroxisome proliferator-activated receptor-gamma mediates bisphenol A inhibition of FSH-stimulated IGF-1, aromatase, and estradiol in human granulosa cells. Environ Health Perspect. 118(3) (2010): p. 400-6.
89. A. Janesick and B. Blumberg, Minireview: PPARgamma as the target of obesogens. J Steroid Biochem Mol Biol. 127(1-2) (2011): p. 4-8.
90. C.J. Hao, X.J. Cheng, H.F. Xia, and X. Ma, The endocrine disruptor diethylstilbestrol induces adipocyte differentiation and promotes obesity in mice. Toxicol Appl Pharmacol. 263(1) (2012): p. 102-10.
91. T.T. Schug, A. Janesick, B. Blumberg, and J.J. Heindel, Endocrine disrupting chemicals and disease susceptibility. J Steroid Biochem Mol Biol. 127(3-5) (2011): p. 204-15.
92. M.N. Jacobs, G.T. Nolan, and S.R. Hood, Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). Toxicol Appl Pharmacol. 209(2) (2005): p. 123-33.
93. H. Watanabe, A. Suzuki, M. Kobayashi, D.B. Lubahn, H. Handa, and T. Iguchi, Similarities and differences in uterine gene expression patterns caused by treatment with physiological and non-physiological estrogens. J Mol Endocrinol. 31(3) (2003): p. 487-97.
94. A. Bouskine, M. Nebout, F. Brucker-Davis, M. Benahmed, and P. Fenichel, Low doses of bisphenol A promote human seminoma cell proliferation by activating PKA and PKG via a membrane G-protein-coupled estrogen receptor. Environ Health Perspect. 117(7) (2009): p. 1053-8.
95. Z. Liu, X. Yu, and Z.A. Shaikh, Rapid activation of ERK1/2 and AKT in human breast cancer cells by cadmium. Toxicol Appl Pharmacol. 228(3) (2008): p. 286-94.

96. K.C. Chitra, C. Latchoumycandane, and P.P. Mathur, Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology. 185(1-2) (2003): p. 119-27.
97. B.T. Akingbemi, R.T. Youker, C.M. Sottas, R. Ge, E. Katz, G.R. Klinefelter, B.R. Zirkin, and M.P. Hardy, Modulation of rat Leydig cell steroidogenic function by di(2-ethylhexyl)phthalate. Biol Reprod. 65(4) (2001): p. 1252-9.
98. B.T. Akingbemi, C.M. Sottas, A.I. Koulova, G.R. Klinefelter, and M.P. Hardy, Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology. 145(2) (2004): p. 592-603.
99. D. Nakamura, Y. Yanagiba, Z. Duan, Y. Ito, A. Okamura, N. Asaeda, Y. Tagawa, C. Li, K. Taya, S.Y. Zhang, H. Naito, D.H. Ramdhan, M. Kamijima, and T. Nakajima, Bisphenol A may cause testosterone reduction by adversely affecting both testis and pituitary systems similar to estradiol. Toxicol Lett. 194(1-2) (2010): p. 16-25.
100. B.H. Yeung, H.T. Wan, A.Y. Law, and C.K. Wong, Endocrine disrupting chemicals: Multiple effects on testicular signaling and spermatogenesis. Spermatogenesis. 1(3) (2011): p. 231-239.
101. P.P. Mathur and S.C. D'Cruz, The effect of environmental contaminants on testicular function. Asian J Androl. 13(4) (2011): p. 585-91.
102. R.J. Aitken and S.D. Roman, Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev. 1(1) (2008): p. 15-24.
103. H. Kabuto, S. Hasuike, N. Minagawa, and T. Shishibori, Effects of bisphenol A on the metabolisms of active oxygen species in mouse tissues. Environ Res. 93(1) (2003): p. 31-5.
104. R.C. Patra, A.K. Rautray, and D. Swarup, Oxidative stress in lead and cadmium toxicity and its amelioration. Vet Med Int. 2011(2011): p. 457327.
105. S. Dhanabalan and P.P. Mathur, Low dose of 2,3,7,8 tetrachlorodibenzo-p-dioxin induces testicular oxidative stress in adult rats under the influence of corticosterone. Exp Toxicol Pathol. 61(5) (2009): p. 415-23.
106. E.W. Wong and C.Y. Cheng, Impacts of environmental toxicants on male reproductive dysfunction. Trends Pharmacol Sci. 32(5) (2011): p. 290-9.
107. C.Y. Cheng, E.W. Wong, P.P. Lie, M.W. Li, L. Su, E.R. Siu, H.H. Yan, J. Mannu, P.P. Mathur, M. Bonanomi, B. Silvestrini, and D.D. Mruk, Environmental toxicants and male reproductive function. Spermatogenesis. 1(1) (2011): p. 2-13.
108. A. Baccarelli and V. Bollati, Epigenetics and environmental chemicals. Curr Opin Pediatr. 21(2) (2009): p. 243-51.
109. M.D. Anway, A.S. Cupp, M. Uzumcu, and M.K. Skinner, Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science. 308(5727) (2005): p. 1466-9.
110. D.C. Dolinoy, D. Huang, and R.L. Jirtle, Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. Proc Natl Acad Sci U S A. 104(32) (2007): p. 13056-61.
111. J.A. Rusiecki, A. Baccarelli, V. Bollati, L. Tarantini, L.E. Moore, and E.C. Bonefeld-Jorgensen, Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect. 116(11) (2008): p. 1547-52.
112. J. Vitku, T. Chlupacova, L. Sosvorova, R. Hampl, M. Hill, J. Heracek, M. Bicikova, and L. Starka, Development and validation of LC–MS/MS method for quantification of bisphenol A and estrogens in human plasma and seminal fluid. Talanta. 140(0) (2015): p. 62-67.
113. R. Hampl, Z. Putz, and L. Stárka, Radioimunologické stanovení dihydrotestosteronu a jeho význam pro laboratorní diagnostiku. Biochem Clin Bohemoslov. 19(1990): p. 157-163.
114. A. Vermeulen, L. Verdonck, and J.M. Kaufman, A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab. 84(10) (1999): p. 3666-72.
115. R.W. Hornung and L.D. Reed, Estimation of Average Concentration in the Presence of Nondetectable Values. Applied Occupational and Environmental Hygiene. 5(1) (1990): p. 46-51.

116. L. Sosvorova, J. Vitku, T. Chlupacova, M. Mohapl, and R. Hampl, Determination of seven selected neuro- and immunomodulatory steroids in human cerebrospinal fluid and plasma using LC-MS/MS. Steroids. 98(2015): p. 1-8.
117. R. Hampl, J. Kubatova, J. Heracek, V. Sobotka, and L. Starka, Hormones and endocrine disruptors in human seminal plasma. Endocr Regul. 47(3) (2013): p. 149-58.
118. R. Hampl, J. Kubatova, V. Sobotka, and J. Heracek, Steroids in semen, their role in spermatogenesis, and the possible impact of endocrine disruptors. Horm Mol Biol Clin Investig. 13(1) (2013): p. 1-5.
119. M.P. Zhao, Y.Z. Li, Z.Q. Guo, X.X. Zhang, and W.B. Chang, A new competitive enzyme-linked immunosorbent assay (ELISA) for determination of estrogenic bisphenols. Talanta. 57(6) (2002): p. 1205-10.
120. N. Kaddar, N. Bendridi, C. Harthe, M.R. de Ravel, A.L. Bienvenu, C.Y. Cuilleron, E. Mappus, M. Pugeat, and H. Dechaud, Development of a radioimmunoassay for the measurement of Bisphenol A in biological samples. Anal Chim Acta. 645(1-2) (2009): p. 1-4.
121. M.S. Bloom, F.S. Vom Saal, D. Kim, J.A. Taylor, J.D. Lamb, and V.Y. Fujimoto, Serum unconjugated bisphenol A concentrations in men may influence embryo quality indicators during in vitro fertilization. Environ Toxicol Pharmacol. 32(2) (2011): p. 319-23.
122. W. Aekplakorn, L.O. Chailurkit, and B. Ongphiphadhanakul, Relationship of serum bisphenol A with diabetes in the Thai population, National Health Examination Survey IV, 2009. J Diabetes,(2014).
123. H.T. Wan, P.Y. Leung, Y.G. Zhao, X. Wei, M.H. Wong, and C.K. Wong, Blood plasma concentrations of endocrine disrupting chemicals in Hong Kong populations. J Hazard Mater. 261(2013): p. 763-9.
124. J. Sajiki, K. Takahashi, and J. Yonekubo, Sensitive method for the determination of bisphenol-A in serum using two systems of high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 736(1-2) (1999): p. 255-61.
125. K. Inoue, M. Wada, T. Higuchi, S. Oshio, T. Umeda, Y. Yoshimura, and H. Nakazawa, Application of liquid chromatography-mass spectrometry to the quantification of bisphenol A in human semen. J Chromatogr B Analyt Technol Biomed Life Sci. 773(2) (2002): p. 97-102.
126. M. Katayama, Y. Matsuda, K.I. Shimokawa, H. Ishikawa, and S. Kaneko, Preliminary monitoring of bisphenol A and nonylphenol in human semen by sensitive high performance liquid chromatography and capillary electrophoresis after proteinase K digestion. Analytical Letters. 36(12) (2003): p. 2659-2667.
127. H. Fukata, H. Miyagawa, N. Yamazaki, and C. Mori, Comparison of Elisa- and LC-MS-Based Methodologies for the Exposure Assessment of Bisphenol A. Toxicol Mech Methods. 16(8) (2006): p. 427-30.
128. J. Vitku, L. Sosvorova, T. Chlupacova, R. Hampl, M. Hill, V. Sobotka, J. Heracek, M. Bicikova, and L. Starka, Differences in bisphenol A and estrogen levels in the plasma and seminal plasma of men with different degrees of infertility. Physiological Reseach. 64 (Suppl. 2)(2015): p. S303-S311.
129. J. Knez, R. Kranvogl, B.P. Breznik, E. Voncina, and V. Vlaisavljevic, Are urinary bisphenol A levels in men related to semen quality and embryo development after medically assisted reproduction? Fertil Steril. 101(1) (2014): p. 215-221 e5.
130. D.K. Li, Z. Zhou, M. Miao, Y. He, J. Wang, J. Ferber, L.J. Herrinton, E. Gao, and W. Yuan, Urine bisphenol-A (BPA) level in relation to semen quality. Fertil Steril. 95(2) (2011): p. 625-30 e1-4.
131. J.D. Meeker, S. Ehrlich, T.L. Toth, D.L. Wright, A.M. Calafat, A.T. Trisini, X. Ye, and R. Hauser, Semen quality and sperm DNA damage in relation to urinary bisphenol A among men from an infertility clinic. Reprod Toxicol. 30(4) (2010): p. 532-9.
132. A.E. Goldstone, Z. Chen, M.J. Perry, K. Kannan, and G.M. Louis, Urinary bisphenol A and semen quality, the LIFE Study. Reprod Toxicol. 51C(2014): p. 7-13.
133. T.H. Lassen, H. Frederiksen, T.K. Jensen, J.H. Petersen, U.N. Joensen, K.M. Main, N.E. Skakkebaek, A. Juul, N. Jorgensen, and A.M. Andersson, Urinary bisphenol A levels in young

men: association with reproductive hormones and semen quality. Environ Health Perspect. 122(5) (2014): p. 478-84.
134. J. Mendiola, N. Jorgensen, A.M. Andersson, A.M. Calafat, X. Ye, J.B. Redmon, E.Z. Drobnis, C. Wang, A. Sparks, S.W. Thurston, F. Liu, and S.H. Swan, Are environmental levels of bisphenol a associated with reproductive function in fertile men? Environ Health Perspect. 118(9) (2010): p. 1286-91.
135. T. Galloway, R. Cipelli, J. Guralnik, L. Ferrucci, S. Bandinelli, A.M. Corsi, C. Money, P. McCormack, and D. Melzer, Daily bisphenol A excretion and associations with sex hormone concentrations: results from the InCHIANTI adult population study. Environ Health Perspect. 118(11) (2010): p. 1603-8.
136. E.J. Kim, D. Lee, B.C. Chung, H. Pyo, and J. Lee, Association between urinary levels of bisphenol-A and estrogen metabolism in Korean adults. Sci Total Environ. 470-471(2014): p. 1401-7.
137. X. Liu, M. Miao, Z. Zhou, E. Gao, J. Chen, J. Wang, F. Sun, W. Yuan, and D.K. Li, Exposure to bisphenol-A and reproductive hormones among male adults. Environ Toxicol Pharmacol. 39(2) (2015): p. 934-41.
138. J.D. Meeker, A.M. Calafat, and R. Hauser, Urinary bisphenol A concentrations in relation to serum thyroid and reproductive hormone levels in men from an infertility clinic. Environ Sci Technol. 44(4) (2010): p. 1458-63.
139. Q. Zhou, M. Miao, M. Ran, L. Ding, L. Bai, T. Wu, W. Yuan, E. Gao, J. Wang, G. Li, and D.K. Li, Serum bisphenol-A concentration and sex hormone levels in men. Fertil Steril. 100(2) (2013): p. 478-82.
140. W. Zhuang, K. Wu, Y. Wang, H. Zhu, Z. Deng, L. Peng, and G. Zhu, Association of serum bisphenol-A concentration and male reproductive function among exposed workers. Arch Environ Contam Toxicol. 68(1) (2015): p. 38-45.
141. T. Trdan Lusin, R. Roskar, and A. Mrhar, Evaluation of bisphenol A glucuronidation according to UGT1A1*28 polymorphism by a new LC-MS/MS assay. Toxicology. 292(1) (2012): p. 33-41.
142. X. Zhang, H. Chang, S. Wiseman, Y. He, E. Higley, P. Jones, C.K. Wong, A. Al-Khedhairy, J.P. Giesy, and M. Hecker, Bisphenol A disrupts steroidogenesis in human H295R cells. Toxicol Sci. 121(2) (2011): p. 320-7.
143. P. Wisniewski, R.M. Romano, M.M. Kizys, K.C. Oliveira, T. Kasamatsu, G. Giannocco, M.I. Chiamolera, M.R. Dias-da-Silva, and M.A. Romano, Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis. Toxicology. 329(2015): p. 1-9.
144. A. Tohei, S. Suda, K. Taya, T. Hashimoto, and H. Kogo, Bisphenol A inhibits testicular functions and increases luteinizing hormone secretion in adult male rats. Exp Biol Med (Maywood). 226(3) (2001): p. 216-21.
145. P. Grandjean, C. Gronlund, I.M. Kjaer, T.K. Jensen, N. Sorensen, A.M. Andersson, A. Juul, N.E. Skakkebaek, E. Budtz-Jorgensen, and P. Weihe, Reproductive hormone profile and pubertal development in 14-year-old boys prenatally exposed to polychlorinated biphenyls. Reprod Toxicol. 34(4) (2012): p. 498-503.
146. A.H. Giwercman, A. Rignell-Hydbom, G. Toft, L. Rylander, L. Hagmar, C. Lindh, H.S. Pedersen, J.K. Ludwicki, V. Lesovoy, M. Shvets, M. Spano, G.C. Manicardi, D. Bizzaro, E.C. Bonefeld-Jorgensen, and J.P. Bonde, Reproductive hormone levels in men exposed to persistent organohalogen pollutants: a study of inuit and three European cohorts. Environ Health Perspect. 114(9) (2006): p. 1348-53.
147. L. Hagmar, J. Bjork, A. Sjodin, A. Bergman, and E.M. Erfurth, Plasma levels of persistent organohalogens and hormone levels in adult male humans. Arch Environ Health. 56(2) (2001): p. 138-43.
148. J. Sagel, J.H. Levine, R.S. Mathur, G. Rosebrock, J. Gonzalez, C. de Villier, and R.M. Nair, Plasma steroid concentrations in patients with hypopituitarism and Kallman's syndrome: effects of testosterone replacement therapy. Clin Endocrinol (Oxf). 17(3) (1982): p. 223-31.

149. J. Peretz, R.K. Gupta, J. Singh, I. Hernandez-Ochoa, and J.A. Flaws, Bisphenol A impairs follicle growth, inhibits steroidogenesis, and downregulates rate-limiting enzymes in the estradiol biosynthesis pathway. Toxicol Sci. 119(1) (2011): p. 209-17.
150. S. Chouhan, S.K. Yadav, J. Prakash, S. Westfall, A. Ghosh, N.K. Agarwal, and S.P. Singh, Increase in the expression of inducible nitric oxide synthase on exposure to bisphenol A: a possible cause for decline in steroidogenesis in male mice. Environ Toxicol Pharmacol. 39(1) (2015): p. 405-16.
151. J. Vitku, L. Starka, M. Bicikova, M. Hill, J. Heracek, L. Sosvorova, and R. Hampl, Endocrine disruptors and other inhibitors of 11beta-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition. J Steroid Biochem Mol Biol,(2014).
152. R. Hampl, M. Pohanka, M. Hill, and L. Starka, The content of four immunomodulatory steroids and major androgens in human semen. J Steroid Biochem Mol Biol. 84(2-3) (2003): p. 307-16.
153. V. Chmielewski, F. Drupt, and R. Morfin, Dexamethasone-induced apoptosis of mouse thymocytes: prevention by native 7alpha-hydroxysteroids. Immunol Cell Biol. 78(3) (2000): p. 238-46.
154. S. Niro, O. Hennebert, and R. Morfin, New insights into the protective effects of DHEA1). Horm Mol Biol Clin Investig. 4(1) (2010): p. 489-98.
155. R. Hampl, M. Hill, I. Sterzl, and L. Starka, Immunomodulatory 7-hydroxylated metabolites of dehydroepiandrosterone are present in human semen. J Steroid Biochem Mol Biol. 75(4-5) (2000): p. 273-6.
156. U. Ahsan, Z. Kamran, I. Raza, S. Ahmad, W. Babar, M.H. Riaz, and Z. Iqbal, Role of selenium in male reproduction - a review. Anim Reprod Sci. 146(1-2) (2014): p. 55-62.
157. M.I. Camejo, L. Abdala, G. Vivas-Acevedo, R. Lozano-Hernandez, M. Angeli-Greaves, and E.D. Greaves, Selenium, copper and zinc in seminal plasma of men with varicocele, relationship with seminal parameters. Biol Trace Elem Res. 143(3) (2011): p. 1247-54.
158. A.E. Omu, M.K. Al-Azemi, M. Al-Maghrebi, C.T. Mathew, F.E. Omu, E.O. Kehinde, J.T. Anim, M.A. Oriowo, and A. Memon, Molecular basis for the effects of zinc deficiency on spermatogenesis: An experimental study in the Sprague-dawley rat model. Indian J Urol. 31(1) (2015): p. 57-64.

11 Seznam vlastních prací

(Jana Vítků, rodné příjmení Kubátová)

a) Impaktované se vztahem k tématu

1. Hampl R., **Kubátová J.**, Stárka L.: Steroids and endocrine disruptors – History, recent state of art and open questions, J Steroid Biochem Mol Biol (2014) In press, available online. IF = 3.628. Příloha V

2. **Vitku J.**, Starka L., Bicikova M., Hill M., Heracek J., Sosvorova L., Hampl R.: Endocrine disruptors and other inhibitors of 11beta-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition. J Steroid Biochem Mol Biol (2014) In press, available online. IF = 3.628. Příloha VI

3. Sosvorova L., **Vitku J.**, Chlupacova T., Mohapl M., Hampl R.: Determination of seven selected neuro- and immunomodulatory steroids in human cerebrospinal fluid and plasma. Steroids, 98, 1 – 8 (2014). IF = 2.639. Příloha VII

4. **Vitku J.**, Chlupacova T., Sosvorova L., Hampl R., Hill M., Heracek J., Bicikova M., Starka L.: Development and validation of LC-MS/MS method for quantification of bisphenol A and estrogens in human plasma and seminal fluid. Talanta, 140, 62-7 (2015). IF = 3.545. Příloha VIII

5. **Vitku J.**, Sosvorova L., Chlupacova T., Hampl R., Hill M., Sobotka V., Heracek J., Bicikova M., Starka L.: Differences in bisphenol A and estrogen levels in the plasma and seminal plasma of men with different degrees of infertility, Phys Res, 64 (Suppl. 2) S303 - S311 (2015). IF = 1.293. Příloha IX

6. **Vitku J.**, Heracek J., Sosvorova L., Chlupacova T., Hampl R., Hill M., Sobotka V., Bicikova M., Starka L.: The impact of bisphenol A and polychlorinated biphenyls on spermatogenesis and steroidogenesis: two biological fluids in men from an infertility clinic. Environment International, odesláno do tisku. Příloha X

b) Neimpaktované se vztahem k tématu

7. Hampl R., **Kubatova J.**, Heracek J., Sobotka V., Starka L.: Hormones and Endocrine disruptors in human seminal plasma, Endocr Regul, 47(3), 149 – 158 (2013). Příloha I

8. Hampl R., **Kubatova J.**, Sobotka V., Heráček J.: Steroids in semen, their role in spermatogenesis, and the possible impact of endocrine disruptors, Horm Mol Biol Clin Invest, 13(1), 1 – 5 (2013). Příloha II

9. **Kubátová J.**, Stárka L.: Endokrinní disruptory a jejich vliv na spermatogenezi a testikulární steroidogenezi. Diabetologie, metabolismus, endokrinologie, výživa, 16(2), 102 – 107 (2013). Příloha III

10. **Kubátová J.**, Stárka L., Bičíková M., Hampl R.: Endokrinní disruptory – sílící hrozba pro lidskou populaci i volně žijící organismy. Lékařské listy, 11, 14 – 16 (2013). Příloha IV

c) Impaktované bez vztahu k tématu

11. Sramkova M., Duskova M., **Vitku J**., Vcelak J., Matucha P., Bradnova O., de Coreiro J., Starka L.: Levels of adipokines and some steroids during the menstrual cycle. Physiol Res. 64(Suppl. 2) (2015): p. S147-S154. IF = 1.293

12. Sosvorova L., Mohapl M., Hill M., Starka L., Bicikova M., **Vitku J.**, Kanceva R., Bestak J., Hampl R.: Steroid hormones and homocysteine in the outcome of patients with normal pressure hydrocephalus. Physiol Res. 64(Suppl. 2) (2015): p. S227-S236. IF = 1.293

13. Sosvorova L., Mohapl M., Vcelak J., Hill M., **Vitku J**., Hampl R.: The impact of selected cytokines in the follow-up of normal pressure hydrocephalus. Physiol Res. 64(Suppl. 2) (2015): p. S283-S290. IF = 1.293

14. Macova L., Sosvorova L., **Vitku J**., Bicikova M., Hill M., Zamrazilova H., Sedlackova B., Starka L.: Steroid hormones related to 11β-hydroxysteroid dehydrogenase type 1 in treated obesity. Physiol Res. 64(Suppl. 2) (2015): p. S121-S133. IF = 1.293

15. Bicikova M., Duskova M., **Vitku J.**, Kalvachova B., Ripova D., Mohr P., Starka L.: Vitamin D in anxiety and affective disorders. Physiol Res. 64(Suppl. 2) (2015): p. S101-S103. IF = 1.293

16. Sosvorova L., Hill M., Mohapl M., **Vitku J**., Hampl R.: Steroid hormones in prediction of normal pressure hydrocephalus. J Steroid Biochem Mol Biol. 152(2015): p. 124-32. IF = 3.628

17. Sosvorova L., Vcelak J., Mohapl M., **Vitku J**., Bicikova M., Hampl R.: Selected pro- and anti-inflammatory cytokines in cerebrospinal fluid in normal pressure hydrocephalus. Neuro Endocrinol Lett. 35(7) (2014): p. 586-93. IF = 0.799

18. Sosvorova L., Bestak J., Bicikova M., Mohapl M., Hill M., **Kubatova J.**, Hampl R.: Determination of homocysteine in cerebrospinal fluid as an indicator for surgery treatment in patients with hydrocefalus. Physiol Res. 63(4) (2014): p. 521-7. IF = 1.293

19. Paskova A., Parizek A., Hill M., Velikova M., **Kubatova J**., Duskova M., Adamcova K., Koucky M., Simjak P., Cerny A., Starka L.: Steroid metabolome in the umbilical cord: is it necessary to differentiate between arterial and venous blood? Physiol Res. 63(1) (2014): p. 115-26. IF = 1.293

20. Duskova M., Simunkova K., Hill M., Velikova M., **Kubatova J**., Kancheva L., Kazihnitkova H., Hruskovicova H., Pospisilova H., Racz B., Salatova M., Cirmanova V., Kralikova E., Starka L., Parizek A.: Chronic cigarette smoking alters circulating sex

hormones and neuroactive steroids in premenopausal women. Physiol Res. 61(1) (2012): p. 97-111. IF = 1.293

21. Hill M., Paskova A., Kanceva R., Velikova M., **Kubatova J.**, Kancheva L., Adamcova K., Mikesova M., Zizka Z., Koucky M., Sarapatkova H., Kacer V., Matucha P., Meloun M., Parizek A.: Steroid profiling in pregnancy: a focus on the human fetus. J Steroid Biochem Mol Biol. 139(2014): p. 201-22. IF = 3.628

22. Hill M., Vrbikova J., Zarubova J., Kancheva R., Velikova M., Kancheva L., **Kubatova J.**, Duskova M., Marusic P., Parizek A., Starka L.: The steroid metabolome in lamotrigine-treated women with epilepsy. Steroids. 76(12) (2011): p. 1351-7. IF = 2.829

23. Hill M., Parizek A., Cibula D., Kancheva R., Jirasek J.E., Jirkovska M., Velikova M., **Kubatova J.**, Klimkova M., Paskova A., Zizka Z., Kancheva L., Kazihnitkova H., Zamrazilova L., Starka L.: Steroid metabolome in fetal and maternal body fluids in human late pregnancy. J Steroid Biochem Mol Biol. 122(4) (2010): p. 114-32. IF = 2.886

24. Hill M., Zarubova J., Marusic P., Vrbikova J., Velikova M., Kancheva R., Kancheva L., **Kubatova J**., Duskova M., Zamrazilova L., Kazihnitkova H., Simunkova K., Starka L.: Effects of valproate and carbamazepine monotherapy on neuroactive steroids, their precursors and metabolites in adult men with epilepsy. J Steroid Biochem Mol Biol. 122(4) (2010): p. 239-52. IF = 2.886

d) Neimpaktované bez vztahu k tématu

25. Duskova M., Sosvorova L., **Vitku J**., Jandikova H., Racz B., Chlupacova T., De Coreiro J., Starka L.: Changes in the concentrations of corticoid metabolites - the effect of stress, diet and analytical method. Prague Med Rep. 116(4) (2015): p. 268-278.

26. Dušková M., Šimůnková K., Hill M., Velíková M., **Kubátová J**., Hruškovičová H., Pospíšilová H., Rácz B., Cirmanová V., Králíková E., Stárka L.: Cigarette smoking and progesterone and androgen metabolites in premenopausal women. Hormone Molecular Biology and Clinical Investigation. 6(3) (2011): p. 259.

27. Hill M., Parizek A., Velikova M., **Kubatova J.**, Kancheva R., Duskova M., Simunkova K., Klimkova M., Paskova A., Zizka Z., Jirasek J.E., Jirkovska M., Starka L.: The distribution of placental oxidoreductase isoforms provides different milieus of steroids influencing pregnancy in the maternal and fetal compartment. Horm Mol Biol Clin Investig. 4(3) (2010): p. 581-600

28. Hill M., Vrbikova J., Zarubova J., Vcelakova H., Duskova M., Kancheva R., **Kubatova J.**, Starka L.: Sulphates of 3beta-hydroxy-5-ene steroids in women with epilepsy. Prague Med Rep. 111(2) (2010): p. 111-26

e) Abstrakta se vztahem k tématu

Autor ústního sdělení:

- Vliv vybraných endokrinních disruptorů na lidskou spermatogenezi. Studentská vědecká konference Výživa, potraviny a zdraví, Praha, 2.12.2015
- Endokrinní disruptory v semenné tekutině. Pražský sexuologicko-andrologický mezinárodní kongres, Senohraby, 21.11.2015
- Rozdíly v hladinách bisfenolu A a estrogenů v plasmě a seminální tekutině u různě neplodných mužů, Imunoanalýza 2015, Lubochňa – Vyšné Krátke, Slovensko, 8. – 12. 6. 2015
- Endokrinní disruptory - hrozba pro naši populaci? Imunoanalýza 2014, Ľubochňa – Vyšné Krátke, Slovensko, 9. - 13. 6. 2014
- Fytoestrogeny jako endokrinní disruptory ve vztahu k funkci štítné žlázy. Analýza organických látek v životním prostředí. Valtice, 14. -16. 10. 2013
- Význam 11βhydroxysteroidní dehydrogenázy v problematice endokrinních disruptorů. Imunoanalýza 2013, Ľubochňa – Vyšné Krátke, Slovensko, 24. -28. 6. 2013
- Vliv vybraných endokrinních disruptorů na aktivitu 11βhydroxysteroidní dehydrogenázy v testes. 14. Studentská vědecká konference, Praha, 23. 5. 2013
- A New Possible Mechanism of Endocrine Disruptor Action in Testes. 15th International Congress on Hormonal Steroids and Hormones & Cancer, Kanazawa, Japonsko, 15. -17. 11. 2012
  (cena za prezentaci)
- Vliv endokrinních disruptorů na vývoj mléčné žlázy a vznik rakoviny prsu. Imunoanalýza 2012, Lubochňa, SR, 25. - 29. 6. 2012
- Endocrine disruptors and their impact on human spermatogenesis. 4th Czech and International Congress of Andrology, Štiřín, 12. -14. 4. 2012

Spoluautor ústního sdělení:

- Stanovení steroidních hormonů v neobvyklých matricích, Imunoanalýza 2015, Lubochňa – Vyšné Krátke, Slovensko, 8. – 12. 6. 2015
- Vývoj LC-MS/MS metodiky na stanovení steroidních hormonů v biologickém materiálu. Ľubochňa – Vyšné Krátke, Slovensko, 9. - 13. 6. 2014
- Význam stanovení 11beta-hydroxysteroidní dehydrogenázy. Imunoanalýza 2013, Ľubochňa – Vyšné Krátke, Slovensko, 24. - 28. 6. 2013
- Vývoj metodiky pro stanovení aktivity 11β-HSD 1 v lidském séru. Imunoanalýza 2013, Ľubochňa – Vyšné Krátke, Slovensko, 24. - 28. 6. 2013

Autor posterového sdělení:

- Determination of Estrogen and BPA Levels in Human Plasma and Seminal Fluid Using LC-MS/MS in Men with Different Degree of Infertility. HPLC 2015, Ženeva, Švýcarsko, 21. – 25. 6. 2015 (cena – poster mezi 20 nejlepšími)
- Development of LC-MS/MS Method for Determination of Bisphenol A and Estrogens in Human Plasma. IFBLS 2014 - The 31st World Congress of Biomedical Laboratory Science, Taipei, Taiwan, 3. – 7. 10. 2014

- Differences between plasmatic concentrations of 7-hydroxylated metabolites of DHEA in healthy men and oligospermic or asthenospermic men. ICN 2014 (International Congress of Neuroendocrinology 2014), Sydney, Austrálie, 17. – 20. 8. 2014
- Development of LC-MS/MS Method for Determination of 11β-hydroxysteroid Dehydrogenase Activity in Infertile Men. RBSC 2013 (Regional European Biomedical Laboratory Science Congress, and the 4th Greek Medical Laboratory Technologists Conference), Athény, Řecko, 5. – 7. 12. 2013
- Vývoj nové metodiky pro stanovení aktivity 11β-hydroxysteroidní dehydrogenázy u mužů s různým stupněm neplodnosti. XXXVI. Endokrinologické dny, Plzeň, 10. – 12. 10. 2013
- The Development of LC-MS/MS Method for Estimation of 11β-hydroxysteroid Dehydrogenase Activity in Men with Different Degree of Infertility. 9th Balaton Symposium on High-Performance Separation Methods, Siofok, Maďarsko, 4. – 6. 9. 2013
- The effects of selected endocrine disruptors on the activity of testicular 11β-hydroxysteroid dehydrogenase in men with various degree of infertility. ENDO 2013, San Francisco, Kalifornie, USA, 15. – 18. 6. 2013

Spoluautor posterového sdělení:

- Development of LC-MS/MS Method for Determination of Bisphenol A and Estrogens in Human Plasma and Seminal Fluid. HPLC 2015, Ženeva, Švýcarsko, 21. – 25. 6. 2015
- Significance of determination of 11beta-hydroxysteroid dehydrogenase. ENDO 2013, San Francisco, Kalifornie, USA, 15. – 18. 6. 2013
- Vliv vybraných endokrinních disruptorů na lidskou spermatogenezi. 58. výroční konference České urologické společnosti ČLS JEP, Ostrava, 31. 10. -1. 11. 2012

PŘÍLOHA I

Hampl R, Kubatova J, Heracek J, Sobotka V, Starka L: Hormones and Endocrine disruptors in human seminal plasma, Endocr Regul, 47(3), 149 – 158 (2013).

doi:10.4149/endo_2013_03_149

Hormones and endocrine disruptors in human seminal plasma

[1]Hampl R, [1]Kubatova J, [2]Heracek J, [2]Sobotka V, [1]Starka L

[1] Institute of Endocrinology and [2]Department of Urology, Third Faculty of Medicine, Charles University, Prague, Czech Republic
E-mail: rhampl@endo.cz

Seminal plasma represents a unique environment for maturation, nutrition, and protection of male germ cells from damaging agents. It contains an array of organic as well as inorganic chemicals, encompassing a number of biologically and immunologically active compounds, including hormones. Seminal plasma contains also various pollutants transferred from outer environment known as endocrine disruptors. They interfere with hormones at the receptor level, act as inhibitors of their biosynthesis, and affect hormone regulation.

In this minireview, the main groups of hormones detected in seminal plasma are summarized. Seminal gonadal steroids were investigated mostly with aim to use them as biomarkers of impaired spermatogenesis (sperm count, motility, morphology). Concentrations of hormones in the seminal plasma often differ considerably from the blood plasma levels in dependence on their origin. In some instances (dihydrotestosterone, estradiol), their informative value is higher than determination in blood.

Out of peptide hormones detected in seminal plasma, peptides of transforming growth factor beta family, especially antimullerian hormone, and oligopeptides related to thyrotropin releasing hormone have the high informative value, while assessment of seminal gonadotropins and prolactin does not bring advantage over determination in blood.

Though there is a large body of information about the endocrine disruptors' impact on male reproduction, especially with their potential role in decline of male reproductive functions within the last decades, there are only scarce reports on their presence in seminal plasma. Herein, the main groups of endocrine disruptors found in seminal plasma are reviewed, and the use of their determination for investigation of fertility disorders is discussed.

Key words: hormone, steroid, peptide hormones, seminal plasma, endocrine disruptors

Introduction

Seminal plasma is a unique environment for maturation, nutrition, and protection of male germ cells from damaging agents and aggressive reactive oxygen species (ROS). From the immunological point of view, it protects sperms from infection and, at the same time, it helps to overcome immunological barrier of the female organism, enabling thus penetration of sperms into the ovum at conception.

Seminal plasma contains an array of biochemical substances, including variety of enzymes and other proteins, substrates and intermediates of biochemical pathways, nutrients, phospholipids and immunologically active components, as well as a number of other biologically active compounds, including hormones.

Corresponding Author: Prof. RNDr. Richard Hampl, DrSc., Institute of Endocrinology Narodni 8, 116 94 Praha 1, Czech Republic; phone: +420 224905289; fax: +420 224905325; e-mail: rhampl@endo.cz.

The constituents present in the seminal plasma enable to compensate hostile biochemical and vaginal, mostly acidic, environment. From the electrolyte balance and buffering capacity maintaining point of view, the important components of seminal plasma are inorganic substances as ions and trace elements (Owen and Katz 2005). In addition, various chemicals from the outer environment may via the blood circulation enter the seminal plasma and affect its properties and function.

In the following text, we will provide a list of main classes of hormones detected in the seminal plasma and their origin and role in the spermatogenesis. Their determination as potential biomarkers for laboratory diagnostics of disorders of male reproductive function will be discussed, too. Finally, a survey of environmental contaminants adversely influencing male reproduction and fertility, known as endocrine disrupting chemicals (EDCs, endocrine disruptors, EDs) found in seminal fluid, will be summarized.

Hormones in seminal plasma

Steroid hormones

It is not surprising that first of all most attention has been paid to hormonal steroids, i.e. gonadal androgens and estrogens. The first reports regarding the sex steroids concentrations in seminal plasma appeared as early as in the seventies (for survey of the older literature see Hampl et al. 2003).

Sex steroids

The range of reported androgen and estrogen levels in seminal plasma is very broad and reflects the methodological development during the last decades. More recent data of seminal and blood plasma concentrations of four major androgens and estradiol in normospermic men (with appropriate references) is shown in Table 1.

As evident, seminal and blood plasma concentrations of sex hormones differ considerably. While testosterone in seminal plasma is in average seven times lower than in blood, the levels of dihydrotestosterone are comparable in both fluids, and estradiol is even higher in semen. It reflects the origin of male sex steroids, while testosterone derives essentially from the accessory sex glands, dihydrotestosterone mainly comes from epididymis (Le Lannou et al. 1980). In the case of estradiol, it reflects high aromatase activity in semen (Carreau et al. 2009). Low concentrations of dihydrotestosterone correlated very well with impaired spermatogenesis (Facchinetti et al. 1987; Zalata et al. 1995; Schwartz et al. 1997; Laudat et al. 1998; Luboshitzky et al. 2002) as did high levels of estradiol (Singer et al. 1987; Santiemma et al. 1991; Bujan et al. 1993; Luboshitzky et al. 2002; Zhang et al. 2010), and therefore, these steroids were recommended as biomarkers for assessment of semen quality.

On the other hand, the data on testosterone in normospermic versus oligo-, astheno- or azoospermic men were rather controversial and did not bring improvement over determination in blood plasma (Facchinetti et al. 1987; Singer et al. 1987; Bujan et al. 1993; Zalata et al. 1995; Laudat et al. 1998; Luboshitzky et al. 2002; Zhang et al. 2010).

Immunomodulatory steroids

The data regarding the seminal dehydroepiandrosterone and its sulfate (DHEA/S) are scarce (Bujan et al. 1993; Hampl et al. 2003). Their presence in seminal plasma may be of interest with respect to the immunological properties of DHEA and its 7-hydroxylating metabolites as immunomodulatory steroids, counteracting the excessive action of glucocorticoids (Morfin et al. 2000; Hampl et al. 2003). Cortisol is also present in the seminal fluid but its concentration is about one fourth of the physiological plasma levels (59 – 176 nmol/l) (Abbaticchio et al. 1981; Brotherton 1990; Hampl et al. 2003). Cortisol influences the biosynthesis of testicular androgens through its receptors in Leydig cells (Hu et al. 2008). In addition, it belongs to the factors regulating cytokine composition (Rogatsky and Ivashkiv 2006; Seshadri et al. 2011). The local balance between biologically active cortisol and inactive cortisone is maintained by the enzyme 11β-hydroxysteroid dehydrogenase (Types 1 and 2), present also in the seminal fluid (Nacharaju et al. 1997). As it will be shown below, this enzyme is one of the targets of EDCs. With respect to its immunomodulatory effects, cortisol represents a potential biomarker of male fertility impairment of (auto)immune origin in seminal plasma.

Peptide hormones

Steroids are not only hormones present in seminal plasma. It contains a set of hormonally active peptides, some of which may serve as biomarkers of male fertility disorders. To the latter group, prostatic oligopeptides of thyroid releasing hormone (TRH)-like family, insulin-like growth factors (IGFs), some peptides of transforming growth factor beta (TGF-β) family as TGF-β itself, inhibins, activins,

Table 1

Average concentrations of four major androgens and estradiol in seminal and blood plasma of normospermic men

Steroid	Seminal plasma reported levels (nmol/l)	Reference	Blood plasma reported levels (nmol/l)	Reference
Te	2.39	Moreno-Escallon et al. 1982	16.5	Moreno-Escallon et al. 1982
	2.18	Bujan et al. 1993	23.9	Bujan et al. 1993
	0.70	Zalata et al. 1995	15.3	Zalata et al. 1995
	1.39	Luboshitzky et al. 2002	18.7	Luboshitzky et al. 2002
	4.60	Zhang et al. 2010	16.4	Zhang et al. 2010
DHT	1.92	Facchinetti et al. 1987	1.66	Schwarzt et al. 1997
	1.14	Zalata et al. 1995		
	1.22	Laudat et al. 1998		
	1.22	Schwarzt et al. 1997		
AD	0.53	Facchinetti et al. 1987	4.00	Wilson et al. 1998
	0.93	Laudat et al. 1998		
DHEAS	1400	Bujan et al. 1993	6850	Bujan et al. 1993
E2	0.60	Bujan et al. 1993	0.096	Bujan et al. 1993
	0.24	Luboshitzky et al. 2002	0.086	Luboshitzky et al. 2002
	0.26	Zhang et al. 2010	<0.18	Wilson et al. 1998

Abbreviations: Te – testosterone; DHT – dihydrotestosterone; DHEAS – dehydroepiandrosterone sulfate; AD – androstenedione, E2 – 17β-estradiol

and antimullerian hormone (AMH) can be included. Since seminal plasma is very rich in proteins, it is often difficult to separate peptide hormones from the protein matrix, which makes it difficult to determine them.

TGF-β related peptides: TGF-β, inhibins, activins, and AMH

The content of TGF-β in seminal plasma belongs to the highest ones measured in biological fluids. It mostly originates from seminal vesicles. Seminal TGF-β is one of the principal stimulating agents in the post-coital inflammatory response, involved in the induction of immune tolerance to seminal antigens (see Introduction). From this point of view, its determination may be important in the targeted therapy of immune-based infertility disorders (Robertson et al. 2002).

Inhibins and activins are homo- or heterodimers formed by combinations of two peptide subunits, α and β. Inhibin immunoreactivity has been found in human seminal plasma as early as in 1981 (Scott and Burger 1981), and inhibin B was later tested as an indicator of the number of released spermatozoa. It was concluded that inhibin secretion into the ejaculate is a marker of the functional activity of the seminiferous tubule, but due to its very broad concentration range in seminal plasma it is not a better marker than the serum hormone (Anderson 2001; Deffieux and Antoine 2003).

AMH is specifically produced by Sertoli cells since the 5th week of gestation under influence of follicle-stimulating hormone (FSH) and later also of testosterone, but it has no feed-back effect on the major hormones of the gonadal axis. As such, it is believed to be a better marker of Sertoli cells development and activity than FSH, and its concentration in seminal plasma correlates well with the number of mature spermatozoa (Deffieux and Antoine 2003). AMH determination in the seminal plasma of men with non-obstructive azoospermia may be used as a marker of the existence of testicular spermatozoa when intracytoplasmic sperm injection is considered (Rey 2000). However, it is completely absent in all cases of obstructive azoospermia (Mostafa et al. 2007).

TRH-like oligopeptides

Though thyroid hormones influence testicular function through their receptors in both Sertoli and Leydig

cells (Maran 2003) and there are many reports dealing with the association of semen quality and the circulating thyroid hormones (Rajender et al. 2011), the reports on thyroid hormone content in seminal plasma are scarce (Landau et al. 1983; Eiler and Armstrong-Backus 1987). In seminal plasma, the presence of 5-deiodase, a key factor regulating local supply of biologically active triiodothyronine (T3), as an essential factor in the testicular paracrine function, has been documented (Brzezinska-Slebodzinska et al. 2000). The highest 5'-deiodinase activity in the male reproductive system is expressed in the epididymis (Anguiano et al. 2008) and local generation of T3 could be associated with the development and function of epididymis and/or spermatozoa maturation.

Seminal plasma is rich in tripeptide related to hypothalamic thyrotropin releasing hormone (TRH, thyreoliberin, p-Glu-His-Pro-NH_2), called fertilization promoting peptide (FPP, pGlu-Glu-ProNH_2), produced by prostate. Under its influence uncapacitated spermatozoa undergo accelerated capacitation and become able to fertilize. As such, it belongs to potential biomarkers of the male fertility (Fraser 1998; Fraser and Adeoya-Osiguwa 2001).

Insulin-like peptides

Insulin-like growth factor 1 (IGF-1) present in seminal plasma is another potential biomarker of male fertility disorders. As a paracrine acting agent, it is involved in the development of male germ cells acting via its receptor in the plasma membrane of human sperm (Sanchez-Luengo et al. 2005). It has been shown that its concentration in seminal plasma significantly correlates with the percentage of morphologically normal spermatozoa (Glander et al. 1996) as well as with the total sperm count (Colombo and Naz 1999). Peptide relaxin, with a similar chemical structure and mechanism of action as insulin, also belongs to this group of hormones present in seminal fluid. It is synthesized in the prostate and released into the seminal fluid. Among many other actions, it stimulates sperm motility and thus increases the sperm penetration into oocytes. With respect to its pleiotropic effects, it has not been suggested as suitable biomarker of the male fertility (Bani 1997).

Gonadotropins and prolactin

Both lutropin (LH) and follitropin (FSH) have been detected and measured in the human seminal plasma as early as in 1970ties. While FSH concentrations in seminal plasma were almost the same as in blood plasma, LH levels were about three times higher. The attempts were made to use these as markers of impaired spermatogenesis, but it was concluded that their measurement in semen does not bring advantage over determination in blood (Shirai et al. 1975, Schoenfeld et al. 1978, Mladenovic et al. 1993).

Prolactin is transported from blood to accessory sex organs and then released into the seminal fluid. Its concentrations in seminal plasma are close to those in the blood, though both levels need not correlate (Gonzales et al. 1989; Arowojolu et al. 2004). Seminal prolactin has been reported to be positively associated with sperm motility and negatively with sperm count (Arowojolu et al. 2004). However, these findings were not confirmed by others and therefore, its determination in seminal plasma cannot be recommended as an alternative to determination in blood plasma.

Other peptide hormones

The presented list of peptide hormones found in seminal plasma is far not complete, but we tried to focus only to those which may serve as biochemical markers of male fertility. Angiotensin II and calcitonin belong to the group of peptide hormones found in seminal plasma (Fraser 1998). Herein, we may mention at least extra-hypothalamic oxytocin which is also produced locally within the testis, and possibly also in the epididymis and prostate, from where it is released to seminal fluid. It is involved in the pathophysiology of prostatic disease (Nicholson and Whittington 2007) and its possible role in respect to the fate of the semen following ejaculation was also discussed (Ivell et al.1997).

Endocrine disruptors in seminal plasma

EDCs – their origin and role in male reproduction

According to the United States Environmental Protection Agency (USEPA), EDCs or EDs are defined as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (Gore 2010). This definition encompasses a large list of industrial materials, plastics and plasticizers, pesticides, fungicides, plant constituents, metals, and many other anthropogenic materials including drugs and nutrients and, last but not least, contraceptives coming to in drain from urine. Some

authors include to EDCs also natural substances like phytoestrogens.

Many of these compounds have been detected in body fluids (blood plasma, urine) and semen. From the chemical point of view, following major groups of EDCs have been found in the seminal fluid: a) diesters of 1,2-benzenedicarboxylic acid (phthalates); b) polychlorinated biphenyls, like phthalates used in industrial applications; c) other organochlorine compounds, including the most dangerous persistent pesticide known as DDT and its major metabolite, DDE; d) 4,4'-(propane-2,2-diyl)diphenol known as bisphenol A, widely used in various plastics and

Disruptor type	Chemical	Method	Studied group	Reference
Phthalates	Various phthalate esters	GC-MS	Infertile men vs. healthy controls	Rozati et al. 2002
	DHPH and its metabolite MEPH	HPLC	Healthy men	Mazzeo et al. 2007
	Various phthalate esters	Reverse phase GC	Infertile vs. fertile men	Pant et a. 2008
	DEHP, MEHP	UPLC-MS/MS	Healthy volunteers	Han et al. 2009
PCB	Various PCB isomers	GC	Infertile men vs. healthy controls	Bush et al. 1986
	Various PCB isomers	GC-MS	Random population sample	Schlebush et al. 1989
	Various PCB isomers	GC	Random population sample	Stachel et al. 1989
	Various PCB isomers	GC-MS	Infertile men vs. healthy controls	Rozati et al. 2002
	PCB isomers 118, 138, 153, 180	GC	Men according to sperm quality	Dallinga et al. 2002
	PCB isomers 118, 126, 153	GC-MS	Random population sample	Pflieger-Bruss et al. 2006
	PCB isomers 49, 153, 180	GC	Men attending IVF program	Younglai et al. 2002
Other organochlorine compounds	p,p´-DDE	GC	Infertile men vs. healthy controls	Bush et al. 1986
	HBC, BHC, DDT and metabolites	GC	Random population sample	Stachel et al. 1989
	12 different chlorine hydrocarbons	GC	Men attending IVF programme	Wagner et al. 1990
	p,p´-DDE	GC	Men attending IVF programme	Younglai et al. 2002
Organobromine compounds	Polybrominated diphenyl esters	GC-MS	Population sample from one Chinese region	Liu et al. 2012
Perfluorochemicals	13 perfluorinated organic compounds PFOA, PFOS	HPLC LC-MS/MS	Urban and rural population in Sri Lanka Large male population sample	Guruge et al. 2005 Raymer et al. 2012
Bisphenols	Bisphenol A	LC-MS	Evaluation of analytical method	Inoue et al. 2002
Dioxins	Dioxin and dioxin like chemicals: TCDD	GC-MS	Veterans from Vietnam	Schecter et al. 1996

Abbreviations: Methods: GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry, LC-MS/MS, liquid chromatography-tandem mass spectrometry, UPLC-MS/MS, ultra-performance liquid chromatography and tandem mass spectrometry
Chemicals: a-BHC, hexachlorocyclohexane; p,p´-DDE, dichlorodiphenyldichloroethylene; DDT, 1,1,1-trichlor-2,2-bis(4-chlorfenyl) ethane; DEHP, di(2-ethylhexyl)phthalate; HCB, Hexachlorobenzene; MEHP, mono(2-ethylhexyl)phthalate; PCB, polychlorinated biphenyls; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; TCDD, dioxin congener 2,3,7,8-tetrachloro-dibenzo(b,e) (1,4)dioxin

epoxy resins; e) other polychlorinated heterocyclic compounds derived from dibenzo(b,e)(1,4)dioxin known as dioxins, persistent environmental pollutants accumulated in the food chain; and f) perfluorinated or polybrominated organic compounds mostly used in agriculture. The list of these compounds with their chemical names is provided in Table 2.

A large array of studies has brought evidence that EDCs influence semen quality as measured usually by sperm volume, motility, and morphology. The evidence for relationship between semen quality and exposure to EDCs is limited to adult males, though semen quality in adulthood may be affected by EDCs exposure during earlier stages of life and even by transgeneration transfer (Hauser et al. 2010).

An important risk factor responsible for the low sperm quality is an impairment of seminal antioxidant system, usually measured as activities of antioxidant enzymes (Sharma and Agarwal 1996; Deepinder et al. 2008). Therefore, it could be presumed that EDCs influence antioxidant status, but so far, we have found only little information on association of seminal plasma EDCs with sperm antioxidant capacity. However, further studies, especially in humans, are needed (Yousef et al. 2003; Jin et al. 2011; Atig et al. 2012).

The main biochemical effects of EDCs consist in their interaction with hormonal receptors as agonists or antagonists. It concerns both membrane receptors, typical for peptide and protein hormones, and intracellular receptors, characteristic for steroids. EDCs act as inhibitors of key enzymes of hormone biosynthesis and elimination. This is typical for the steroid hormones. Finally, EDCs may interfere with endocrine axes at various levels (gonadal, adrenal, thyroid).

The presence of EDCs in the environment is often put into connection with observed decline of semen quality in the last 5-6 decades with regard to sperm count, motility, and morphology. The main outcomes have been published in official documents of the World Health Organization (WHO) (WHO Library Cataloguing-in-Publication Data 2012) as well as in the already cited monography (Gore 2010), but their discussion would largely exceed this minireview. In the present study, we focus only to EDCs in semen.

EDCs in seminal plasma

How EDCs may influence the male reproduction and development has recently been reviewed by Hauser et al. (2010). Epidemiologic studies as well as animal experiments summarized therein have brought a large amount of evidence that exposure to EDCs may lead to an impairment of spermatogenesis and their high levels in blood and urine were associated with fertility disorders.

There is, however, relatively scarce information available on the relationship of EDCs in seminal plasma with the above mentioned disorders. In other words, whether their determination might help in the search for the origin of fertility problems and observed disorders. In the present paper, we tried to review the available data of the main groups of EDCs detected and measured in seminal plasma. Their survey is in Table 2. The cited works include: 1) analytical papers, some of them are the first reports evidencing the presence of EDCs in seminal plasma (Stachel et al. 1989; Wagner et al. 1990; Inoue et al. 2002; Mazzeo et al. 2007; Liu et al. 2012); 2) attempts to establish occurrence of selected EDCs in seminal plasma from general male population or in men exposed to various environmental pollutants (Schlebush et al. 1989; Schecter et al. 1996; Younglai et al. 2002; Guruge et al. 2005; Han et al. 2009); and 3) to find out, what extent of seminal EDCs is associated with fertility disorders, with particular respect to sperm characteristics – sperm count, motility, and morphology (Bush et al. 1986; Dallinga et al. 2002; Rozati et al. 2002; Pant et al. 2008).

As the latter is concerned, the main outcomes were as follows: high PCBs levels were detected in seminal plasma of infertile men but not in controls (Bush et al. 1986; Rozati et al. 2002) and were inversely related to the sperm motility (Dallinga et al. 2002). Infertile men had also higher levels of DDE. Similarly, concentration of phthalate esters was significantly higher in infertile men than in controls (Rozati et al. 2002; Pant et al. 2008). On the other hand, only the report dealing with perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) in seminal plasma did not revealed any effect of perfluorinated organic pollutants on the sperm concentration and motility (Raymer et al. 2012). It is evident that more studies are needed to determine the relation between the other groups of EDCs found in seminal plasma and impaired spermatogenesis.

As mentioned above, an important constituent of the seminal plasma is cortisol with its immunosuppressive properties. Its local concentration in semen is regulated by the isoenzymes of 11β-hydroxysteroid dehydrogenase (Nacharaju et al. 1997). Some EDCs such as phthalate esters, many different pesticides, and phytoestrogens are potent inhibitors of both enzyme types and thus may strongly influence the glucocorticoid action in the testis (Ohshima et al. 2005; Ma et al. 2011).

Conclusion

Taking into account their role in male reproduction, hormones determined in seminal plasma may serve as important biomarkers of male fertility disorders, in particular of impaired spermatogenesis, in some instances even better than blood plasma hormones. The seminal plasma contains also environmental chemicals, acting as endocrine disruptors. They reflect the exposure of men to ubiquitous pollutants, which, of course, differ considerably according to regions, occupation and generally global situation. There is surprisingly little information on their occurrence in seminal plasma. We suggest that their simultaneous determination in seminal plasma, along with hormones, the biosynthesis and mechanism of action of which is under influence of these chemicals, would bring new insight on the problems associated with male fertility.

Acknowledgements

This work was supported by the Grant No. 13369-4 of the Internal Grant Agency of the Czech Ministry of Health.

References

Abbaticchio G, Giorgino R, Urago M, Gattuccio F, Orlando G, Janni A: Hormones in the seminal plasma. Cortisol. Acta Eur Fertil 12, 239-244, 1981.

Anderson RA: Clinical studies: inhibin in the adult male. Mol Cell Endocrinol 180, 109-116, 2001. http://dx.doi.org/10.1016/S0303-7207(01)00510-X

Anguiano B, Aranda N, Delgado G, Aceves C: Epididymis expresses the highest 5'-deiodinase activity in the male reproductive system: kinetic characterization, distribution, and hormonal regulation. Endocrinology 149, 4209-4217, 2008. http://dx.doi.org/10.1210/en.2007-1679

Arowojolu AO, Akinloye O, Shittu OB: Serum and seminal plasma prolactin levels in male attenders of an infertility clinic in Ibadan. J Obstet Gynaecol 14, 306-309, 2004. http://dx.doi.org/10.1080/01443610410001660931

Atig F, Raffa M, Ali HB, Abdelhamid K, Saad A, Ajina M: Altered antioxidant status and increased lipid peroxidation in seminal plasma of tunisian infertile men. Int J Biol Sci 8, 139-149, 2012. http://dx.doi.org/10.7150/ijbs.8.139

Bani D: Relaxin: a pleiotropic hormone. Gen Pharmacol 28, 13-22, 1997. http://dx.doi.org/10.1016/S0306-3623-(96)00171-1

Brotherton J: Cortisol and transcortin in human seminal plasma and amniotic fluid as estimated by modern specific assays. Andrologia 22, 197-204, 1990. Brzezinska-Slebodzinska E, Slebodzinski AB, Kowalska K: Evidence for the presence of 5'-deiodinase in mammalian seminal plasma and for the increase in enzyme activity in the prepubertal testis. Int J Androl 23, 218-224, 2000. http://dx.doi.org/10.1046/j.1365-2605.2000.00233.x

Bujan L, Mieusset R, Audran F, Lumbroso S, Sultan C: Increased oestradiol level in seminal plasma in infertile men. Hum Reprod 8, 74-77, 1993.

Bush B, Bennett AH, Snow JT: Polychlorobiphenyl congeners, p,p'-DDE, and sperm function in humans. Arch Environ Contam Toxicol 15, 333-341, 1986. http://dx.doi.org/10.1007/BF01066399

Carreau S, Delalande C, Galeraud-Denis I: Mammalian sperm quality and aromatase expression. Microsc Res Tech 72, 552-557, 2009. http://dx.doi.org/10.1002/jemt.20703

Colombo JB, Naz RK: Modulation of insulin-like growth factor-1 in the seminal plasma of infertile men. J Androl 20, 118-125, 1999.

Dallinga JW, Moonen EJ, Dumoulin JC, Evers JL, Geraedts JP, Kleinjans JC: Decreased human semen quality and organochlorine compounds in blood. Hum Reprod 17, 1973-1979, 2002. http://dx.doi.org/10.1093/humrep/17.8.1973

Deffieux X, Antoine JM: Inhibins, activins and anti-Mullerian hormone: structure, signalling pathways, roles and predictive value in reproductive medicine. Gynecol Obstet Fertil 31, 900-911, 2003. http://dx.doi.org/10.1016/j.gyobfe.2003.08.012

Deepinder F, Cocuzza M, Agarwal A: Should seminal oxidative stress measurement be offered routinely to men presenting for infertility evaluation? Endocr Prac 14, 484-491 2008. http://dx.doi.org/10.4158/EP.14.4.484

Eiler H, Armstrong-Backus CS: Passage of exogenous L-thyroxine and triiodothyronine into bovine ejaculate. Am J Vet Res 48, 439-443, 1987.

Facchinetti F, Comitini G, Genazzani A, Bakalakis C, Genazzani AR, Loche S: Seminal fluid androgen levels in infertile patients. Int J Fertil 32, 157-161, 1987.

Fraser LR: Fertilization promoting peptide: an important regulator of sperm function in vivo? Rev Reprod 3, 151-154, 1998. http://dx.doi.org/10.1530/ror.0.0030151

Fraser LR, Adeoya-Osiguwa SA: Fertilization promoting peptide--a possible regulator of sperm function in vivo. Vitam Horm 63, 1-28, 2001. http://dx.doi.org/10.1016/S0083-6729(01)63001-2

Glander HJ, Kratzsch J, Weisbrich C, Birkenmeier G: Insulin-like growth factor-I and alpha 2-macroglobulin in seminal plasma correlate with semen quality. Hum Reprod 11, 2454-2460, 1996. http://dx.doi.org/10.1093/oxfordjournals.humrep.a019136

Gonzales GF, Garcia-Hjarles M, Velazquez G, Coyotupa J: Seminal prolactin and its relationship to sperm motility in men. Fertil Steril 51, 498-503, 1989.

Gore AC: Introduction to endocrine-disrupting chemicals. In: Endocrine disrupting chemicals: From basic research to clinical practice (Ed. AC Gore), pp. 3-8, Humana Press Inc, Totowa, NJ 2010.

Guruge KS, Taniyasu S, Yamashita N, Wijeratna S, Mohotti KM, Seneviratne HR, Kannan K, Yamanaka N, Miyazaki S: Perfluorinated organic compounds in human blood serum and seminal plasma: a study of urban and rural tea worker populations in Sri Lanka. J Environ Monit 7, 371-377, 2005. http://dx.doi.org/10.1039/b412532k

Hampl R, Pohanka M, Hill M, Starka L: The content of four immunomodulatory steroids and major androgens in human semen. J Steroid Biochem Mol Biol 84, 307-316, 2003. http://dx.doi.org/10.1016/S0960-0760(03)00044-X

Han SW, Lee H, Han SY, Lim DS, Jung KK, Kwack SJ, Kim KB, Lee BM: An exposure assessment of di-(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DBP) in human semen. J Toxicol Environ Health 72, 1463-1469, 2009. http://dx.doi.org/10.1080/15287390903212972

Hauser R, Barthold JS, Meeker JD: Epidemiologic evidence on the relationship between environmental endocrine disruptors and male reproductive and developmental health. In: Endocrine disrupting chemicals: From basic research to clinical practice, Ed. AC Gore, pp. 225-251, Humana Press Inc, Totowa, NJ 2010.

Hu GX, Lian QQ, Lin H, Latif SA, Morris DJ, Hardy MP, Ge RS: Rapid mechanisms of glucocorticoid signaling in the Leydig cell. Steroids 73, 1018-1024, 2008. http://dx.doi.org/10.1016/j.steroids.2007.12.020

Inoue K, Wada M, Higuchi T, Oshio S, Umeda T, Yoshimura Y, Nakazawa H: Application of liquid chromatography-mass spectrometry to the quantification of bisphenol A in human semen. J Chromatogr B Analyt Technol Biomed Life Sci 773, 97-102, 2002. http://dx.doi.org/10.1016/S1570-0232(02)00101-0

Ivell R, Balvers M, Rust W, Bathgate R, Einspanier A: Oxytocin and male reproductive function. Adv Exp Med Biol 424, 253-264, 1997. http://dx.doi.org/10.1007/978-1-4615-5913-9_47

Jin Y, Wang L, Ruan M, Liu J, Yang Y, Zhou C, Xu B, Fu Z: Cypermethrin exposure during puberty induces oxidative stress and endocrine disruption in male mice. Chemosphere 84, 124-130, 2011. http://dx.doi.org/10.1016/j.chemosphere.2011.02.034

Landau B, Singer R, Shindel B, Laor J, Sagiv M, Barnet M: Triiodothyronine and tetraiodothyronine in human semen prior to, and following, treatment with thyroid extracts. Experientia 39, 544-545, 1983. http://dx.doi.org/10.1007/BF01965199

Laudat A, Guechot J, Palluel AM: Seminal androgen concentrations and residual sperm cytoplasm. Clin Chim Acta 276, 11-18, 1998. http://dx.doi.org/10.1016/S0009-8981(98)00090-4

Le Lannou D, Massart C, Chambon Y, Nicol M, Allannic H: Testosterone and 5alpha-dihydrotestosterone concentrations in human seminal plasma. Int J Androl 3, 502-506, 1980. http://dx.doi.org/10.1111/j.1365-2605.1980.tb00138.x

Liu PY, Zhao YX, Zhu YY, Qin ZF, Ruan XL, Zhang YC, Chen BJ, Li Y, Yan SS, Qin XF, Fu S, Xu XB: Determination of polybrominated diphenyl ethers in human semen. Environ Int 42, 132-137, 2012. http://dx.doi.org/10.1016/j.envint.2011.05.011

Luboshitzky R, Shen-Orr Z, Herer P: Seminal plasma melatonin and gonadal steroids concentrations in normal men. Arch Androl 8, 225-232, 2002.
http://dx.doi.org/10.1080/01485010252869324

Ma X, Lian QQ, Dong Q, Ge RS: Environmental inhibitors of 11β-hydroxysteroid dehydrogenase type 2. Toxicology 285, 83-89, 2011. http://dx.doi.org/10.1016/j.tox.2011.04.007

Maran RR: Thyroid hormones: their role in testicular steroidogenesis. Arch Androl 49, 375-388, 2003.

Mazzeo P, Di Pasquale D, Ruggieri F, Fanelli M, D'Archivio AA, Carlucci G: HPLC with diode-array detection for the simultaneous determination of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in seminal plasma. Biomed Chromatogr 21, 1166-1171, 2007. http://dx.doi.org/10.1002/bmc.870

Mladenovic I, Genbacev O, Movsesijan M, Micic S, Papic N: Gonadotropins (FSH and LH) and testosterone in human male serum and seminal plasma. Acta Eur Fertil 24, 79-85, 1993.

Moreno-Escallon B, Ridley AJ, Wu CH, Blasco L: Hormones in seminal plasma. Arch Androl 9, 127-134, 1982. http://dx.doi.org/10.3109/01485018208990230

Morfin R, Lafaye P, Cotillon AC, Nato F, Chmielewski V, Pompon D: 7alpha-hydroxy-dehydroepiandrosterone and immune response. Ann N Y Acad Sci 917, 971-982, 2000. http://dx.doi.org/10.1111/j.1749-6632.2000.tb05464.x

Mostafa T, Amer MK, Abdel-Malak G, Nsser TA, Zohdy W, Ashour S, El-Gayar D, Awad HH: Seminal plasma anti-Müllerian hormone level correlates with semen parameters but does not predict success of testicular sperm extraction (TESE). Asian J Androl 9, 265-270, 2007. http://dx.doi.org/10.1111/j.1745-7262.2007.00252.x

Nacharaju VL, Muneyyirci-Delale O, Khan N: Presence of 11 beta-hydroxysteroid dehydrogenase in human semen: evidence of correlation with semen characteristics. Steroids 62, 311-314, 1997. http://dx.doi.org/10.1016/S0039-128X(96)00225-5

Nicholson HD, Whittington K: Oxytocin and the human prostate in health and disease. Int Rev Cytol 263, 253-286, 2007. http://dx.doi.org/10.1016/S0074-7696(07)63006-X

Ohshima M, Ohno S, Nakajin S: Inhibitory effects of some possible endocrine-disrupting chemicals on the isozymes of human 11beta-hydroxysteroid dehydrogenase and expression of their mRNA in gonads and adrenal glands. Environ Sci 12, 219-230, 2005.

Owen DH, Katz DF: A review of the physical and chemical properties of human semen and the formulation of a semen simulant. J Androl 26, 459-469, 2005. http://dx.doi.org/10.2164/jandrol.04104

Pant N, Shukla M, Kumar Patel D, Shukla Y, Mathur N, Kumar Gupta Y, Saxena DK: Correlation of phthalate exposures with semen quality. Toxicol Appl Pharmacol 231, 112-116, 2008. http://dx.doi.org/10.1016/j.taap.2008.04.001

Pflieger-Bruss S, Hagemann S, Korner W, Hanf V, Kohn FM, Muller C, Schill WB: Effects of single non-ortho, mono-ortho, and di-ortho chlorinated biphenyls on human sperm functions in vitro. Reprod Toxicol 21, 280-284, 2006. http://dx.doi.org/10.1016/j.reprotox.2005.09.011

Rajender S, Monica MG, Walter L, Agarwal A: Thyroid, spermatogenesis, and male infertility. Front Biosci (Elite Ed.) 3, 843-855, 2011.

Raymer JH, Michael LC, Studabaker WB, Olsen GW, Sloan CS, Wilcosky T, Walmer DK: Concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) and their associations with human semen quality measurements. Reprod Toxicol 33, 419-427, 2012. http://dx.doi.org/10.1016/j.reprotox.2011.05.024

Rey R: Assessment of seminiferous tubule function (anti-müllerian hormone). Baillieres Best Pract Res Clin Endocrinol Metab 14, 399-408, 2000. http://dx.doi.org/10.1053/beem.2000.0087

Robertson SA, Ingman WV, O'Leary S, Sharkey DJ, Tremellen KP: Transforming growth factor beta-a mediator of immune deviation in seminal plasma. J Reprod Immunol 57, 109-128, 2002. http://dx.doi.org/10.1016/S0165-0378(02)00015-3

Rogatsky I, Ivashkiv LB: Glucocorticoid modulation of cytokine signaling. Tissue Antigens 68, 1-12, 2006. http://dx.doi.org/10.1111/j.1399-0039.2006.00599.x

Rozati R, Reddy PP, Reddanna P, Mujtaba R: Role of environmental estrogens in the deterioration of male factor fertility. Fertil Steril 78, 1187-1194, 2002. http://dx.doi.org/10.1016/S0015-0282(02)04389-3

Sanchez-Luengo S, Fernandez PJ, Romeu A: Insulin growth factors may be implicated in human sperm capacitation. Fertil Steril 83, 1064-1066, 2005. http://dx.doi.org/10.1016/j.fertnstert.2004.12.003

Santiemma V, Rosati P, Fazzi V, Bolelli GF, Guerzoni C, Fabbrini A: Seminal estrone, estrone sulfate, and estradiol-17 beta levels in fertile and infertile males. Arch Androl 26, 129-134, 1991. http://dx.doi.org/10.3109/01485019108987635

Schecter A, McGee H, Stanley JS, Boggess K, Brandt-Rauf P: Dioxins and dioxin-like chemicals in blood and semen of American Vietnam veterans from the state of Michigan. Am J Ind Med 30, 647-6554, 1996. http://dx.doi.org/10.1002/(SICI)1097-0274(199612)30:6<647::AID-AJIM1>3.0.CO;2-O

Schlebusch H, Wagner U, van der Ven H, al-Hasani S, Diedrich K, Krebs D: Polychlorinated biphenyls: the occurrence of the main congeners in follicular and sperm fluids. J Clin Chem Clin Biochem 27, 663-667, 1989.

Schoenfeld C, Amelar RD, Dubin L, Numeroff M: Follicle-stimulating hormone, luteinizing hormone, and testosterone levels found in human seminal plasma. Fertil Steril 29, 69-71, 1978.

Schwartz JI, Tanaka WK, Wang DZ, Ebel DL, Geissler LA, Dallob A, Hafkin B, Gertz BJ: MK-386, an inhibitor of 5alpha-reductase type 1, reduces dihydrotestosterone concentrations in serum and sebum without affecting dihydrotestosterone concentrations in semen. J Clin Endocrinol Metab 82, 1373-1377, 1997. http://dx.doi.org/10.1210/jc.82.5.1373

Scott RS, Burger HG: An inverse relationship exists between seminal plasma inhibin and serum follicle-stimulating hormone in man. J Clin Endocrinol Metab 52, 796-803, 1981. http://dx.doi.org/10.1210/jcem-52-4-796

Seshadri S, Bates M, Vince G, Jones DI: Cytokine expression in the seminal plasma and its effects on fertilisation rates in an IVF cycle. Andrologia 43, 378-386, 2011. http://dx.doi.org/10.1111/j.1439-0272.2010.01042.x

Sharma RK, Agarwal A: Role of reactive oxygen species in male infertility. Urology 48, 835-850, 1996. http://dx.doi.org/10.1016/S0090-4295(96)00313-5

Shirai M, Matsuda S, Mitsukawa S, Nakamura M, Yonezawa K: FSH, LH and testosterone levels in human seminal plasma. Tohoku J Exp Med 116, 201-202, 1975. http://dx.doi.org/10.1620/tjem.116.201

Singer R, Sagiv M, Bruchis S, Barnet M, Kaufman H, Servadio C: Total and free testosterone and estradiol in human semen. Int J Fertil 32, 145-148, 1987.

Stachel B, Dougherty RC, Lahl U, Schlosser M, Zeschmar B: Toxic environmental chemicals in human semen: analytical method and case studies. Andrologia 21, 282-291, 1989. http://dx.doi.org/10.1111/j.1439-0272.1989.tb02412.x

Wagner U, Schlebusch H, van der Ven H, van der Ven K, Diedrich K, Krebs D: Accumulation of pollutants in the genital tract of sterility patients. J Clin Chem Clin Biochem 28, 683-688, 1990.

WHO Library Cataloguing-in-Publication Data. State of the science of endocrine disrupting chemicals 2012, (Eds. A Bergman, JJ Heindel, S Jobling, KA, R Kidd, T Zoeller), WHO publications, 2012.

Wilson JD, Foster DW, Kronenberg HM, Larsen PR (Eds): Williams Textbook of Endocrinology, 9th Edition, WB Saunders Co, Philadelphia 1998.

Younglai EV, Foster WG, Hughes EG, Trim K, Jarrell JF: Levels of environmental contaminants in human follicular fluid, serum, and seminal plasma of couples undergoing in vitro fertilization. Arch Environ Contam Toxicol 43, 121-126, 2002. http://dx.doi.org/10.1007/s00244-001-0048-8

Yousef MI, El-Demerdash FM, Al-Salhen KS: Protective role of isoflavones against the toxic effect of cypermethrin on semen quality and testosterone levels of rabbits. J Environ Sci Health B 38, 463-478, 2003. http://dx.doi.org/10.1081/PFC-120021666

Zalata A, Hafez T, Verdonck L, Vermeulen L, Comhaire F: Androgens in seminal plasma: markers of the surface epithelium of the male reproductive tract. Int J Androl 18, 271-277, 1995.

Zhang Q, Bai Q, Yuan Y, Liu P, Qiao J: Assessment of seminal estradiol and testosterone levels as predictors of human spermatogenesis. J Androl 31, 215-220, 2010. http://dx.doi.org/10.2164/jandrol.109.007609

PŘÍLOHA II

Hampl R, Kubatova J, Sobotka V, Heráček J: Steroids in semen, their role in spermatogenesis, and the possible impact of endocrine disruptors, Horm Mol Biol Clin Invest, 13(1), 1 – 5 (2013).

DOI 10.1515/hmbci-2013-0003 — Horm Mol Biol Clin Invest 2013; 13(1): 1–5

Mini Review

Richard Hampl*, Jana Kubátová, Vladimír Sobotka and Jiří Heráček

Steroids in semen, their role in spermatogenesis, and the possible impact of endocrine disruptors

Abstract: The data on hormonal steroids in the human seminal plasma and their role in spermatogenesis are summarized. The seminal steroid levels need not correlate with the blood plasma levels. The recent reports showed that androgen, especially dihydrotestosterone, and the estrogen levels in the seminal fluid may be used as the markers of spermatogenesis impairment. The estradiol concentration in the seminal plasma was higher than in the blood plasma, and its levels were significantly increased in men with impaired spermatogenesis. A good indicator for predicting the normal spermatogenesis, therefore, seems to be the testosterone/estradiol ratio. The seminal plasma also contains significant amounts of cortisol, which influences the androgen biosynthesis through its receptors in the Leydig cells. The local balance between cortisol and inactive cortisone is regulated by 11β-hydroxysteroid dehydrogenase, the activity of which may be affected by the environmental chemicals acting as the endocrine disruptors (EDCs). These compounds are believed to participate in worsening the semen quality – the sperm count, motility, and morphology, as witnessed in the recent last decades. As to the steroids' role in the testis, the EDCs may act as antiandrogens by inhibiting the enzymes of testosterone biosynthesis, as the agonists or antagonists through their interaction with the steroid hormone receptors, or at the hypothalamic-pituitary-gonadal axis. Surprisingly, though the EDCs affect the steroid action in the testis, there is no report of a direct association between the concentrations of steroids and the EDCs in the seminal fluid. Therefore, measuring the steroids in the semen, along with the various EDCs, could help us better understand the role of the EDCs in the male reproduction.

Keywords: endocrine disruptors; steroid in semen; spermatogenesis.

***Corresponding author: Prof. RNDr. Richard Hampl,** DrSc., Institute of Endocrinology, Národní 8, 116 94 Prague, Czech Republic, Phone: +420 224905 289, Fax: +420 224905 325, E-mail: rhampl@endo.cz
Jana Kubátová: Institute of Endocrinology, Prague, Czech Republic
Vladimír Sobotka and Jiří Heráček: Third Faculty of Medicine, Department of Urology, Charles University in Prague, Czech Republic

Steroids in testes – their biosynthesis and role in male reproduction

The seminal plasma represents a unique milieu for the protection and maturation of the sperm cells facilitating their penetration into the ovum at conception. It contains the substrates needed for nutrition, a large array of enzymes and other proteins, phospholipids, and a number of other biologically active compounds including hormones. It is well known that the sex steroids and other steroid hormones, in concert with the nonsteroidal constituents of the seminal fluid, are necessary for functional spermatogenesis. Since the introduction of the sensitive methods of steroid determination in the body fluids in the 1970s, a number of reports have appeared on the steroid concentration in the seminal plasma. The main task was to assess to what extent their determination may help in the diagnosis of the various sperm abnormalities (oligo-, astheno-, azoospermia) in comparison with the determination of their plasma levels and whether the correlation between their concentration in the plasma and semen does exist. The survey of the literature on the steroids in the seminal fluid listed chronologically, together with the reported range of values is shown in Table 1. On the right part of the table, the corresponding levels in the blood plasma are provided for comparison. The range of the reported seminal steroid concentration is very broad, especially in the case of testosterone, which is rather due to the methodology used, than to the differences in the sperm parameters. Table 2, therefore, shows the selected data from the literature of testosterone measurement in both seminal and blood plasma in normospermic men.

As for testosterone, while in most earlier papers the authors did not find significant differences between the normal men and those with impaired spermatogenesis (see Ref. [1–6, 9, 10, 12]), the more recent refinement of the analytical methods revealed a reduction in the testosterone levels in oligo-, astheno-, or azzoospermic men (see Ref. [7, 11–16]) in Table 1). As evident from Table 1, the

Table 1 The reported concentration range of the five hormonal steroids in the human seminal plasma and blood (nmol/L).

Steroid	Seminal fluid	References	Blood plasma or serum	References
Testosterone	0.3–4.6	[1–16]	10–35	[6, 12, 13, 15–17]
Dihydrotestosterone	1.1–1.9	[1, 3–5, 7, 8, 11, 13, 14, 18]	0.87–2.6	[17, 18]
Androstenedione	0.3–2.0	[3, 11, 13]	3.0–5.0	[17]
Estradiol	0.2–0.6	[1, 7, 10, 12, 15, 16, 19]	<0.18	[12, 15, 17]
Cortisol	59–176	[20–22]	140–690	[17]

Table 2 The reported mean testosterone concentrations in the seminal plasma and blood from the normospermic men (nmol/L).

Seminal plasma	Blood plasma or serum	Author, year	Reference
2.39	16.5	Moreno-Escalon et al. 1982	[6]
2.18	23.9	Bujan et al. 1993	[12]
0.70	15.3	Zalata et a. 1995	[13]
1.39	18.7	Luboshitzky et al. 2002	[15]
4.60	16.4	Zhang et al. 2010	[16]

dihydrotestosterone (DHT) levels in the seminal plasma were comparable with those of testosterone, but the reported data did not show significant differences [1, 3–5, 7, 8, 10, 13, 14, 18], and in contrast to testosterone, it was in most instances lower in men with impaired spermatogenesis than in the normospermic men and, therefore, is believed to be an even better marker of sperm quality [10]. The seminal concentrations of the above main androgens usually correlated well with each other, but they did not need to correlate with the respective blood plasma levels [13]. The levels of the testosterone precursor androstenedione in the seminal plasma were similar to testosterone and did not usually correlate with the spermatologic parameters [3, 11, 13]. The estradiol concentration in the seminal plasma was even higher than in the male blood plasma, and its levels were significantly increased in men with impaired spermatogenesis [1, 7, 10, 12, 15, 16, 19]. More recent studies demonstrated that the seminal gonadal steroids, specifically the testosterone/estradiol levels and/or their ratios, may be good indicators for predicting the normal spermatogenesis [16]. Concerning the source of the major male sex steroids in the seminal fluid, it was concluded that testosterone is derived essentially from the accessory sex glands, whereas DHT is mainly of testicular or epididymal origin [5].

The human seminal plasma also contains measurable amounts of cortisol. The reported mean values in the normospermic men varied from 59 to 176 nmol/L [20–22], thus reaching from 11% [20, 22] to 60% [21] of the respective serum levels. It is of special interest in light of the fact that the glucocorticoids influence the testosterone biosynthesis through their receptors in the Leydig cells [23, 24]. As shown below, many environmental compounds known as endocrine disruptors (EDCs) and found in the seminal plasma may affect the actual levels of the biologically active glucocorticoids through their effect on the 11β-hydroxysteroid dehydrogenase enzymes.

Endocrine disruptors, steroids, and effects on spermatogenesis

The large population studies and meta-analyses brought evidence that the quality of semen with regard to the sperm count, motility, and morphology has declined in the last 50–60 years [25–27]. The data from the other studies, however, did not confirm these findings [28–30]. It was concluded that the results must be interpreted with caution due to the great heterogeneity and other bias, geographical and/or ethnical variation, different study designs, and different methodological approaches [31]. These trends have been attributed at least partly to the exposure to the environmental chemicals that act as EDCs, which, among many other effects, influence the steroid actions in the testis [32, 33]. The problematics has been reviewed in the recent monography, summarizing the most important human as well as animal studies [34]. The main groups of the chemicals acting as EDCs include a large list of industrial materials, plastics, pesticides, plant constituents, and many other anthropogenic materials including drugs and nutrient constituents; see Ref. [33–35]. As for the role of the steroids in the testis, the EDCs may principally act in three ways: 1. as antiandrogens by inhibiting one or more enzymes of the testosterone biosynthesis [35]; 2. as agonists or antagonists through their interaction with the steroid hormone receptors – androgen, estrogen, and even glucocorticoid, all of which are present in the testis [23, 24, 36–39]; 3. by affecting the hypothalamo-pituitary-gonadal axis on the central level [40]. In addition to their effects as hormone agonists and antagonists, the EDCs as, e.g., parahydroxybenzoic acid esters (parabens), polychlorinated

biphenyls, or bisphenol A, may damage the sperm DNA, as demonstrated (among others) by the association between the urinary EDCs with the sperm DNA damage measures [41–43]. It would be worth noticing to measure these EDCs in the seminal fluid. As shown in the previous paragraph, the steroids in the seminal fluid influence the quality of semen and, in general, spermatogenesis, as do (usually in the opposite way) the EDCs. The question may be raised whether there is an association between the EDCs and steroid content in the seminal plasma; in other words, whether the EDCs affect the steroid biosynthesis and action in semen. Surprisingly, to the best of our knowledge, there is no report on the direct association between the concentrations of the steroids and the EDCs measured in the seminal fluid. However, as shown below, the EDCs, either from the environment or experimentally administered, possess various effects on the seminal steroid action.

Another group of chemicals with the potential to influence the male fertility are soy phytoestrogens, which are part of the common diet in the East Asian countries. The major representatives of this group are the isoflavones, daidzein and genistein, and the metabolite of the former, equol. They act as week estrogens via their interaction with the estradiol receptors (β type). Their consumption in the South and East Asian countries is often put in connection with the lower incidence of female as well as male cancers [44]. Studies were, therefore, performed on whether their consumption may affect the sperm parameters and, more generally, male reproduction. Though some indications exist that phytoestrogens may alter the reproductive hormones, spermatogenesis, sperm capacitation, and fertility, no unequivocal effects were observed [33, 45–48]. So far, we have not found any report dealing with their residues in the spermatic fluid.

Effects of endocrine disruptors on seminal steroid action

The steroid 5-α-reductase inhibitors, such as finasteride or MK-386, have been used for the treatment of benign prostate hyperplasia or cancer, and also for acne. A clinical study was performed on how the administration of these drugs influences the DHT content in the semen. While finasteride reduced the seminal DHT only slightly, no effect was observed after MK-386 [18]. This points to the importance of the internal milieu represented by the semen for spermatogenesis.

The glucocorticoids present in the seminal plasma, cortisol in humans, and corticosterone in rodents, counteract the androgen action via their receptors in the Leydig cells [23, 24]. In addition, the glucocorticoids are the regulators of an array of pro- and anti-inflammatory cytokines and transcription factors [49–51], many of which are also present in the semen [52, 53]. The local glucocorticoid concentration in the testis is governed by the 11β-hydroxysteroid dehydrogenase isoenzymes. Their expression was demonstrated in the rat Leydig cells [54], and their activity was proven in the human semen [55]. The EDCs as alkylphenols, phthalate esters, phytoestrogens, organotins, and pesticides are potent inhibitors of both the enzyme types and, thus, may strongly influence the glucocorticoid action in the testis [56, 57].

Conclusion and outlooks

We tried to demonstrate that the steroid hormones in the human seminal plasma play an important role in spermatogenesis and may serve as the biomarkers for the assessment of successful fertility. The chemicals from the environment known to act as the EDCs affect considerably the spermatogenesis and reproductive functions in the males. It is rather surprising that there are only a few reports on the levels of the EDCs in the semen [58, 59] and even fewer studies on the association of the EDCs in the seminal plasma and the respective steroid concentrations. We suggest that the measurement of the steroids in seminal fluid, along with the assessment of the various EDCs, is needed to help better understand the role of the EDCs in the male reproduction.

Acknowledgments: This work was supported by Grant Project No. 13369-4 from the Internal Grant Agency of the Czech Ministry of Health.

Received February 11, 2013; accepted April 9, 2013; previously published online May 7, 2013

References

1. Purvis K, Landgren BM, Cekan Z, Diczfalusy E. Indices of gonadal function in the human male. II. Seminal plasma levels of steroids in normal and pathological conditions. Clin Endocrinol (Oxf) 1975;4:247–58.
2. Adamopoulos DA, Lawrence DM, Swyer GI. Determination of testosterone concentration in semen of men with normal or subnormal sperm counts and after vasectomy. Acta Eur Fertil 1976;7:219–25.

3. De Aloysio D, D'Urso N, Giardini G, Nicoletti G, Gori G. Plasma and semen androgens in normospermic and dispermic subjects. Acta Eur Fertil 1978;9:139–44.
4. Pazzagli M, Giusti G, Forti G, Fiorelli G, Menchini-Fabris F, Scarselli GF, Guazzelli R, Conti C, Borrelli D, Cicchi P, Serio M. Seminal plasma levels of testosterone and 5alpha-dihydrotestosterone in azoospermic patients. Clin Endocrinol (Oxf) 1979;11:11–4.
5. Le Lannou D, Massart C, Chambon Y, Nicol M, Allannic H. Testosterone and 5 alpha-dihydrotestosterone concentrations in human seminal plasma. Int J Androl 1980;3:502–6.
6. Moreno-Escallon B, Ridley AJ, Wu CH, Blasco L. Hormones in seminal plasma. Arch Androl 1982;9:127–34.
7. García Díez LC, Gonzalez Buitrago JM, Corrales JJ, Battaner E, Miralles JM. Hormone levels in serum and seminal plasma of men with different types of azoospermia. J Reprod Fertil 1983;67:209–14.
8. Kurniawan E, Tamm J, Volkwein U, Schirren C. Unconjugated 5 alpha-androstan-3 alpha, 17 beta-diol and 5 alpha-androstane-3 beta, 17 beta-diol in normal and pathological human seminal plasma. Comparison with testosterone, 5 alpha-dihydrotestosterone and testosterone-glucosiduronate. Andrologia 1983;5:141–50.
9. Adamopoulos D, Lawrence DM, Vassilopoulos P, Kapolla N, Kontogeorgos L, McGarrigle HH. Hormone levels in the reproductive system of normospermic men and patients with oligospermia and varicocele. J Clin Endocrinol Metab 1984;59:447–552.
10. Singer R, Sagiv M, Bruchis S, Barnet M, Kaufman H, Servadio C. Total and free testosterone and estradiol in human semen. Int J Fertil 1987;32:145–8.
11. Facchinetti F, Comitini G, Genazzani A, Bakalakis C, Genazzani AR, Loche S. Seminal fluid androgen levels in infertile patients. Int J Fertil 1987;32:157–61.
12. Bujan L, Mieusset R, Audran F, Lumbroso S, Sultan C. Increased oestradiol level in seminal plasma in infertile men. Hum Reprod 1993;8:74–7.
13. Zalata A, Hafez T, Verdonck L, Vermeulen L, Comhaire F. Androgens in seminal plasma: markers of the surface epithelium of the male reproductive tract. Int J Androl 1995;18:271–7.
14. Laudat A, Guechot J, Palluel AM. Seminal androgen concentrations and residual sperm cytoplasm. Clin Chim Acta 1998;276:11–8.
15. Luboshitzky R, Shen-Orr Z, Herer P. Seminal plasma melatonin and gonadal steroids concentrations in normal men. Arch Androl 2002;8:225–32.
16. Zhang Q, Bai Q, Yuan Y, Liu P, Qiao J. Assessment of seminal estradiol and testosterone levels as predictors of human spermatogenesis. J Androl 2010;31:215–20.
17. Wilson JD, Foster DW, Kronenberg HM, Larsen PR, editors. Williams textbook of endocrinology, 9th ed. Philadelphia: WB Saunders Co, 1998.
18. Schwartz JI, Tanaka WK, Wang DZ, Ebel DL, Geissler LA, Dallob A, Hafkin B, Gertz BJ. MK-386, an inhibitor of 5alpha-reductase type 1, reduces dihydrotestosterone concentrations in serum and sebum without affecting dihydrotestosterone concentrations in semen. J Clin Endocrinol Metab 1997;82:1373–7.
19. Santiemma V, Rosati P, Fazzi V, Bolelli GF, Guerzoni C, Fabbrini A. Seminal estrone, estrone sulfate, and estradiol-17 beta levels in fertile and infertile males. Arch Androl 1991;26:129–34.
20. Abbaticchio G, Giorgino R, Urago M, Gattuccio F, Orlando G, Jannì A. Hormones in the seminal plasma. Cortisol. Acta Eur Fertil 1981;12:239–44.
21. Brotherton J. Cortisol and transcortin in human seminal plasma and amniotic fluid as estimated by modern specific assays. Andrologia 1990;22:197–204.
22. Hampl R, Pohanka M, Hill M, Stárka L. The content of four immunomodulatory steroids and major androgens in human semen. J Steroid Biochem Mol Biol 2003;84:307–16.
23. Dong Q, Salva A, Sottas CM, Niu E, Holmes M, Hardy MP. Rapid glucocorticoid mediation of suppressed testosterone biosynthesis in male mice subjected to immobilization stress. J Androl 2004;25:973–81.
24. Hu GX, Lian QQ, Lin H, Latif SA, Morris DJ, Hardy MP, Ge RS. Rapid mechanisms of glucocorticoid signaling in the Leydig cell. Steroids 2008;73:1018–24.
25. Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. Br Med J 1992;305:609–13.
26. Auger J, Kunstmann JM, Czyglik F, Jouannet P. Decline in semen quality among fertile men in Paris during the past 20 years. N Engl J Med 1995;332:281–5.
27. Feki NC, Abid N, Rebai A, Sellami A, Ayed BB, Guermazi M, Bahloul A, Rebai T, Ammar LK. Semen quality decline among men in infertile relationships: experience over 12 years in the South of Tunisia. J Androl 2009;30:541–7.
28. Paulsen CA, Berman NG, Wang C. Data from men in greater Seattle area reveals no downward trend in semen quality: further evidence that deterioration of semen quality is not geographically uniform. Fertil Steril 1996;65:1015–20.
29. Fisch H, Goluboff ET, Olson JH, Feldshuh J, Broder SJ, Barad DH. Semen analyses in 1,283 men from the United States over a 25-year period: no decline in quality. Fertil Steril 1996;65:1009–14.
30. Sripada S, Fonseca S, Lee A, Harrild K, Giannaris D, Mathers E, Bhattacharya S. Trends in semen parameters in the northeast of Scotland. J Androl 2007;28:313–9.
31. Merzenich H, Zeeb H, Blettner M. Decreasing sperm quality: a global problem? BMC Public Health 2010;10:24.
32. Mathur PP, D'Cruz SC. The effect of environmental contaminants on testicular function. Asian J Androl 2011;13:585–91.
33. Phillips KP, Tanphaichitr N. Human exposure to endocrine disrupters and semen quality. J Toxicol Environ Health B Crit Rev 2008;11:188–220.
34. Hauser R, Barthold JS, Meeker JD. Epidemiologic evidence on the relationship between environmental endocrine disruptors and male reproductive and developmental health. In: Gore AC, editor. Endocrine disrupting chemicals: from basic research to clinical practice. Totowa, NJ: Humana Press Inc, 2010:225–51.
35. Ye L, Su ZJ, Ge RS. Inhibitors of testosterone biosynthetic and metabolic activation enzymes. Molecules 2011;16:9983–10001.
36. Tabb MM, Blumberg B. New modes of action for endocrine-disrupting chemicals. Mol Endocrinol 2006;20:475–82.
37. Rey RA, Musse M, Venara M, Chemes HE. Ontogeny of the androgen receptor expression in the fetal and postnatal testis: its relevance on sertoli cell maturation and the onset of adult spermatogenesis. Microsc Res Tech 2009;72:787–95.
38. Martin LJ, Tremblay JJ. Nuclear receptors in Leydig cell gene expression and function. Biol Reprod 2010;83:3–14.

39. Cavaco JE, Laurentino SS, Barros A, Sousa M, Socorro S. Estrogen receptors alpha and beta in human testis: both isoforms are expressed. Syst Biol Reprod Med 2009;55:137–44.
40. Schoeters G, Den Hond E, Dhooge W, van Larebeke N, Leijs M. Endocrine disruptors and abnormalities of pubertal development. Basic Clin Pharmacol Toxicol 2008;102:168–75.
41. Meeker JD, Yang T, Ye X, Calafat AM, Hauser R. Urinary concentrations of parabens and serum hormone levels, semen quality parameters, and sperm DNA damage. Environ Health Perspect 2011;119:252–7.
42. Meeker JD, Ehrlich S, Toth TL, Wright DL, Calafat AM, Trisini AT, Ye X, Hauser R. Semen quality and sperm DNA damage in relation to urinary bisphenol A among men from an infertility clinic. Reprod Toxicol 2010;30:532–9.
43. Meeker JD, Hauser R. Exposure to polychlorinated biphenyls (PCBs) and male reproduction. Syst Biol Reprod Med 2010;56:122–31.
44. Adlercreutz H. Phyto-oestrogens and cancer. Lancet Oncol 2002;3:364–73.
45. Messina M. Soybean isoflavone exposure does not have feminizing effects on men: a critical examination of the clinical evidence. Fertil Steril 2010;93:2095–104.
46. Mitchell JH, Cawood E, Kinniburgh D, Provan A, Collins AR, Irvine DS. Effect of a phytoestrogen food supplement on reproductive health in normal males. Clin Sci (Lond) 2001;100:613–8.
47. Giwercman A. Estrogens and phytoestrogens in male infertility. Curr Opin Urol 2011;21:519–26.
48. Cederroth CR, Auger J, Zimmermann C, Eustache F, Nef S. Soy, phyto-oestrogens and male reproductive function: a review. Int J Androl 2010;33:304–16.
49. Rogatsky I, Ivashkiv LB. Glucocorticoid modulation of cytokine signaling. Tissue Antigens 2006;68:1–12.
50. Flammer JR, Rogatsky I. Minireview: glucocorticoids in autoimmunity: unexpected targets and mechanisms. Mol Endocrinol 2011;25:1075–86.
51. Ratman D, Berghe WV, Dejager L, Libert C, Tavernier J, Beck IM, De Bosscher K. How glucocorticoid receptors modulate the activity of other transcription factors: a scope beyond tethering. Mol Cell Endocrinol 2013, in press.
52. Seshadri S, Bates M, Vince G, Jones DI. Cytokine expression in the seminal plasma and its effects on fertilisation rates in an IVF cycle. Andrologia 2011;43:378–86.
53. Qian L, Sun G, Zhou B, Wang G, Song J, He H. Study on the relationship between different cytokines in the semen of infertility patients. Am J Reprod Immunol 2011;66:157–61.
54. Ge RS, Dong Q, Niu EM, Sottas CM, Hardy DO, Catterall JF, Latif SA, Morris DJ, Hardy MP. 11{beta}-Hydroxysteroid dehydrogenase 2 in rat leydig cells: its role in blunting glucocorticoid action at physiological levels of substrate. Endocrinology 2005;146:2657–64.
55. Nacharaju VL, Muneyyirci-Delale O, Khan N. Presence of 11 beta-hydroxysteroid dehydrogenase in human semen: evidence of correlation with semen characteristics. Steroids 1997;62:311–4.
56. Ohshima M, Ohno S, Nakajin S. Inhibitory effects of some possible endocrine-disrupting chemicals on the isozymes of human 11beta-hydroxysteroid dehydrogenase and expression of their mRNA in gonads and adrenal glands. Environ Sci 2005;12:219–30.
57. Ma X, Lian QQ, Dong Q, Ge RS. Environmental inhibitors of 11β-hydroxysteroid dehydrogenase type 2. Toxicology 2011;285:83–9.
58. Rozati R, Reddy PP, Reddanna P, Mujtaba R. Role of environmental estrogens in the deterioration of male factor fertility. Fertil Steril 2002;78:1187–94.
59. Mazzeo P, Di Pasquale D, Ruggieri F, Fanelli M, D'Archivio AA, Carlucci G. HPLC with diode-array detection for the simultaneous determination of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in seminal plasma. Biomed Chromatogr 2007;21:1166–71.

PŘÍLOHA III

Kubátová J, Stárka L: Endokrinní disruptory a jejich vliv na spermatogenezi a testikulární steroidogenezi. Diabetologie, metabolismus, endokrinologie, výživa, 16(2), 102 – 107 (2013).

ENDOKRINNÍ DISRUPTORY A JEJICH VLIV NA SPERMATOGENEZI A TESTIKULÁRNÍ STEROIDOGENEZI

ENDOCRINE DISRUPTORS AND THEIR IMPACT ON SPERMATOGENESIS AND TESTICULAR STEROIDOGENESIS

JANA KUBÁTOVÁ, LUBOSLAV STÁRKA

Endokrinologický ústav, Praha

SOUHRN

Endokrinní disruptory (ED) jsou látky, které ovlivňují endokrinní systém a zasahují tak do důležitých procesů ve vývoji člověka i volně žijících zvířat. Do životního prostředí se dostávají hlavně antropogenní činností a zahrnují velice různorodou skupinu látek, která dosud není kompletní. U volně žijících zvířat jsou již negativní účinky ED na reprodukční soustavu dokázány, účinky na lidský organizmus zatím nejsou tak důkladně prozkoumány. Studium účinků ED je komplexní problém, protože závisí jak na dávce, tak i době expozice, která je kritická v určitých vývojových obdobích. Mohou se objevovat i synergické účinky ED. V přehledu shrnujeme známé mechanizmy působení ED, s důrazem na poznatky o jejich vlivu na spermatogenezi a steroidogenezi ve varlatech.

Klíčová slova: endokrinní disruptory, spermatogeneze, steroidogeneze, mechanizmus působení

SUMMARY

Endocrine disruptors (EDs) are substances that affect endocrine system and may also interfere with the developmental processes of humans and wildlife species. EDs get into environment mainly through anthropogenic activities and include a very heterogeneous group of chemicals, which is not yet complete. Although negative effects of EDs on reproductive system of wildlife species have been confirmed, effects on humans are still not fully understood. Study on endocrine disruptors´ effects is very complex problem because it depends on the dose as well as on the time of exposure which is critical in certain developmental periods. EDs can also work together to produce synergistic effects. The review summarizes known mechanisms of endocrine disruptor action, with particular focus on their impact on spermatogenesis and testicular steroidogenesis.

Key words: endocrine disruptors, spermatogenesis, steroidogenesis, mechanism of action

Úvod

V posledních dekádách se do životního prostředí dostávají antropogenní činností tisíce tun různých chemikálií. O těchto látkách se předpokládalo, že nemají žádnou, či mají jen velmi malou biologickou toxicitu. Dnes nás obklopují běžně v každodenním životě, jsou např. ve vzduchu, vodě, půdě, potravinách, elektronice či plastech a mnoho z nich má schopnost zasahovat do endokrinního systému. Tyto látky se nazývají endokrinní disruptory. Přehled vědomostí o této skupině látek a stanovisko americké Endokrinologické společnosti k této problematice jsou obsaženy v článku Diamanti-Kandarakis a kol. (Diamanti-Kandarakis et al., 2009).

V posledních desetiletích byly také zaznamenány zhoršující se reprodukční funkce u lidí i zvířat. Již v roce 1992 byla publikována rozsáhlá metaanalýza, kde bylo prezentováno postupné snižování počtu spermií u mužů z různých částí světa v průběhu uplynulých padesáti let (Carlsen et al., 1992). Tyto výsledky podpořily i další studie (Auger et al., 1995; Irvine et al., 1996; Swan et al., 1997), naopak některé pokles počtu a kvality spermií neprokázaly (Fisch et al. 1996; Seo et al. 2000; Zvěřina et al. 2002). Byl zjištěn také sekulární pokles hladin testosteronu u mužů (Andersson et al. 2007; Feldman et al. 2002).

Carlsenová (Carlsen et al., 1992) svoje analýzy dávala do souvislosti právě se zvyšujícím se výskytem chemikálií v prostředí. Kromě kvality spermií byl zaznamenán vyšší výskyt nádorů varlat (Adami et al., 1994) a vyšší riziko kryptorchizmu a hypospadie (Chilvers et al., 1984; Paulozzi, 1999). V letech 1973–1992 byl pozorován pokles koncentrace spermií o 2,1 % za rok, pokles motility spermií o 0,6 % za rok a pokles podílu normálních spermií o 0,5 % za rok (Carlsen et al., 1992).

Skakkebaek v roce 2001 navrhl hypotézu, kdy snížená kvalita spermií, nádory varlat a abnormality ve vývoji fetálních varlat by mohly mít společnou kauzální příčinu. Tuto trojici poruch definoval jako syndrom testikulární dysgeneze. Stále častěji se uvažuje, že expozice endokrinním disruptorům je

největším viníkem těchto problémů. U volně žijících zvířat již byly účinky disruptorů na reprodukční soustavu dokázány. Bylo prezentováno, že množství chemikálií přes chlorované sloučeniny (dioxiny, polychlorované bifenyly), pesticidy, herbicidy až po těžké kovy působí jak na dospělá zvířata, tak na jejich mláďata (Diamanti-Kandarakis et al., 2009). Nejnápadněji jsou postiženy reprodukční funkce.

V tomto přehledovém článku jsou shrnuty známé mechanizmy působení ED, s důrazem na poznatky o jejich vlivu na spermatogenezi a steroidogenezi.

Obecné vlastnosti disruptorů

Disruptory jsou chemicky různorodá skupina látek, které vykazují některé zajímavé charakteristiky. Podobně jako hormony mohou účinkovat ve velice malém množství. V některých případech nižší hladiny ED mohou mít ve výsledku větší účinky na cílovou tkáň než vyšší hladiny ED. Mohou také vykazovat netradiční dynamiku dávky a odpovědi, kdy křivky závislosti dávky na odpovědi mohou mít tvar U či invertované U (Diamanti-Kandarakis et al., 2009). Závisí také na tom, za jakého vývojového stádia je organizmus endokrinním disruptorům vystaven.

Počet publikací o ED dramaticky narůstá – nejvíce zájmu přitahuje jejich vztah k funkci štítné žlázy, na druhém místě je fertilita a na dalším pak onkogenní působení, vztah k nástupu a průběhu puberty a nejnověji pak vztah k obezitě, kdy je některými autory připisován ED klíčový vztah k pandemii obezity.

Důležitý v případě disruptorů je také věk v době expozice. Vystavení se působení ED v dospělosti může mít zcela odlišné důsledky než expozice během vývoje. U dospělých je zpravidla potřeba vyšší hladina ED, aby působila toxicky na organizmus. Naopak během vývoje organizmu stačí nízká dávka po kratší dobu a může mít trvalé následky až do dospělosti, kdy už ED dávno v těle není přítomen. Tento koncept je pojmenován „the fetal basis of adult disease" neboli expozice ED při vývoji organizmu je základem nemoci/poruchy v dospělosti (Barker, 2003). Z řady možných mechanizmů zásahu endokrinních disruptorů do systému reprodukčních funkcí jsou nejdůležitější modifikace metabolizmu hormonů, modifikace jejich působení na příslušné receptory a epigenetické vlivy. Vedle rozdílného působení ED v různých vývojových obdobích člověka, zvláště intenzivních ve „vývojových oknech" (intrauterinně, v raném dětství, v pubertě a ve stáří) patří ke zvláštnostem působení ED také latence působení, někdy i transgenerační působení směsí (častý synergizmus) a netradiční závislosti na dávce. Příkladem synergického působení je dieldrin a endosulfan, které působí 100× vyšším estrogenním efektem v kombinaci než samostatně – EC50 = 100 nM (Arnold et al., 1996). Podobně dieldrin působí synergicky i s jinými disruptory (lindan) (Sharma et al., 2010). Jiní autoři (Wade et al., 1997) prokazují, že kooperativní účinek dieldrinu a sulfanu, pokud vůbec existuje, není natolik silný, aby hrál větší roli v ohrožování lidského zdraví.

Do mužských reprodukčních funkcí mohou zasahovat zejména ED s vlastnostmi anti-androgenů nebo estrogenů. K prvým patří např. polychlorované bifenyly, vinklozolin, nebo DDT/DDE, estrogenní vlastnosti jsou zjištěny pro diethystilbestrol, bisfenol A, polychlorované bifenyly, fytoestrogeny, mykoestrogeny, UV-filtry, kadmium nebo arsenik.

U mužů byla prokazována spoluúčast ED na řadě klinických poruch, jako je snížená kvalita spermií (ftaláty, PCB, dioxiny, neperzistentní pesticidy), karcinom zárodečných buněk, anomálie mužské reprodukční soustavy, karcinom prostaty a erektilní dysfunkce (pesticidy, herbicidy, organická rozpouštědla, průmyslové chemikálie, stilbeny, aditiva pro plasty, sirovodík, olovo, methylbromid).

Spermatogeneze

Spermatogeneze je složitý proces, při kterém nezralá zárodečná buňka podstupuje dělení, diferenciaci a meiózu, aby se stala haploidní spermií. Zahrnuje 3 fáze: spermatogoniální fázi, fázi spermatocytů a fázi spermatidovou (Phillips a Tanphaichitr, 2008). V první fázi spermatogonie, relativně nespecializované diploidní zárodečné buňky, ležící na vnitřní straně semenotvorných kanálků (de Rooij a Russell, 2000), podstupují mitózu za vzniku diploidních spermatocytů. V další fázi diploidní spermatocyty projdou dvěma meiotickými děleními, během kterých vzniknou haploidní spermatidy. Z nezralých kulatých spermatid se v poslední fázi, nazývané spermiogeneze, stávají zralé spermie (O'Donnell et al., 2001).

Pro spermatogenezi a funkci testes jsou zásadní Sertoliho buňky, které nasedají na bazální membránu v semenotvorných kanálcích. Mezi sebou jsou spojeny těsnými spojeními neboli „tight junctions" a tvoří tak bariéru mezi krevním řečištěm a varletem (Walker a Cheng, 2005). Sertoliho buňky podporují zrání zárodečných buněk buď přímým kontaktem se zárodečnou buňkou, nebo řízením vnitřního prostředí v semenotvorných kanálcích (Griswold, 1998). Regulovány jsou folikuly stimulujícím hormonem (FSH) z adenohypofýzy a testosteronem, který je produkován Leydigovými buňkami varlat jako odpověď na stimulaci luteinizačním hormonem (LH) z adenohypofýzy (Phillips a Tanphaichitr, 2008). Testosteron působí na Sertoliho buňky prostřednictvím androgenního receptoru a stimuluje syntézu různých proteinů a trofických faktorů ve specifických fázích spermatogeneze (Wang et al., 2009). Mezi trofické faktory parakrinně secernované Sertoliho buňkami patří např. insulin-like growth factor 1 (IGF-1), nerve growth factor (NGF), growth factor derived from glia (GDNF) a stem cell factor (SCF) (Lagos-Cabre a Moreno, 2012).

Proces spermatogeneze začíná pod vlivem steroidních androgenů a FSH v pubertě a pokračuje v průběhu celého života. Proto je muž během celého tohoto období citlivý na změny v životním stylu a také vystavení se různým chemikáliím v životním prostředí (Sharpe, 2010). Neméně důležité je období fetálního vývoje, kdy jsou položeny základy mužských pohlavních orgánů. Změny ve vývoji způsobené ED by mohly mít zásadní vliv na kvalitu spermatogeneze v dospělosti (Sharpe, 2010).

Steroidogeneze v Leydigových buňkách

Steroidogeneze v Leydigových buňkách je řízena LH. Po vazbě LH na receptor spojený s G-proteiny je stimulována tvorba cyklického adenosin monofosfátu (cAMP) (Dufau, 1998). Vyšší hladiny cAMP následně aktivují na cAMP

závislou proteinkinázu A (PKA), která fosforyluje seriny na steroidním akutním regulačním proteinu (StAR), čímž ho zaktivuje (Arakane et al., 1997). StAR společně s periferním typem benzodiazepinového receptoru (PBR) zprostředkovávají přenos cholesterolu od vnější k vnitřní mitochondriální membráně (Hauet et al., 2002; Papadopoulos, 2004; Stocco, 2001). Přenos cholesterolu je prvním a zároveň limitujícím krokem ve steroidogenezi. Jakmile se cholesterol dostane do vnitřní mitochondriální membrány, je konvertován na pregnenolon cytochromem P450scc (CYP11A1). Pregnenolon se pak z mitochondrie přesouvá do hladkého endoplazmatického retikula, kde je pomocí 3β-hydroxysteroidní dehydrogenázy (3βHSD) přeměněn na progesteron. V dalším kroku se uplatní enzym 17α-hydroxyláza/17,20lyáza (CYP17), která katalyzuje přeměnu progesteronu na androstendion a poslední fáze, přeměna na testosteron, je zajištěna 17β-hydroxysteroidní dehydrogenázou (17βHSD) (Payne a Hales, 2004).

Řízení steroidogeneze

Celá signalizační osa je řízena z hypothalamu pulzně vylučovaným gonadotropin-uvolňujícím hormonem = gonadoliberinem (GnRH), který dále působí na adenohypofýzu. Z té se po podnětu vylučují FSH a LH, které se krevním řečištěm dostávají do varlat. LH působí na Leydigovy buňky v intersticiu a stimuluje tvorbu testosteronu a FSH působí přímo na Sertoliho buňky. Uvolňování GnRH je řízeno hypothalamickým peptidem kisspeptinem-1 a jeho receptorem spřaženým s G-proteiny. Kisspeptin je kódován genem KISS1, který je cílem gonadálních steroidů u obou pohlaví (Silveira et al., 2010). Kisspeptinové neurony exprimují estrogenní receptor α (ERα), progesteronový receptor (PR) a androgenní receptor (AR) (Roseweir a Millar, 2009). Je pravděpodobné, že regulace exprese KISS1 genu bude fungovat jako negativní zpětná vazba (Rance, 2009). Dokázáno to bylo např. na samicích myší, kdy po ovariektomii se gen KISS1 exprimoval více a jeho exprese po podání estradiolu zase klesla (Smith et al., 2005). U myší a lidí, kterým chybí kisspeptinový receptor, se objevuje hypogonadotropní hypogonadizmus a s tím související infertilita (de Roux et al., 2003; Seminara et al., 2003).

Účinky ED na spermatogenezi

Kompetice o vazbu na steroidní nukleární receptor

Endokrinní disruptory jsou strukturně podobné mnoha steroidům, účinkují také v nízkých dávkách a mohou mít lipofilní vlastnosti. Proto jsou schopné napodobovat endogenní hormony a napodobovat jejich mechanizmus působení, transport a ukládání ve tkáních. Jejich vlastnosti jim umožňují navázat se na jaderné receptory a tím aktivovat nebo potlačovat jejich funkci. Je známé působení přes estrogenní receptory α i β, androgenní receptor, tyroidní receptor, progesteronový receptor, receptor pro arylované uhlovodíky (AhR), glukokortikoidní či PPAR (peroxisome proliferator-activated receptor) (Schug et al., 2011). Po navázání ED na receptor se komplex přesune do jádra, má ale většinou jiné účinky než přirozený ligand. Např. diethylstilbestrol jakožto syntetický estrogen se váže na estrogenní receptory. Některé geny aktivuje stejně jako endogenní 17β-estradiol (E2), ale u jiných skupin genů se míra aktivity lišila v porovnání s E2 (Watanabe et al., 2003).

Modulace steroidogeneze

Původně bylo působení endokrinních disruptorů vysvětlováno jen vazbou na nukleární steroidní receptory. Dnes již víme, že mohou působit i na jiných úrovních, mimo jiné mohou narušovat steroidní biosyntézu a metabolizmus (Diamanti-Kandarakis et al., 2009). Negativní účinky disruptorů byly pozorovány v *in vitro* i *in vivo* studiích.

Např. ftaláty ovlivňují steroidogenezi v Leydigových buňkách v závislosti na stádiu fetálního vývoje. Podávání diethylhexyl ftalátu (DEHP) potkanům dva týdny postnatálně má za následek snížení aktivity 17βHSD a hladin testosteronu v Leydigových buňkách. Na druhou stranu podávání DEHP dospělým potkanům steroidogenezi v Leydigových buňkách neovlivňuje (Akingbemi et al., 2001).

Dioxiny většinu, ne-li všechny své účinky na organizmus uskutečňují přes vazbu na AhR. Komplex dioxin-AhR se následně přesune z cytosolu do jádra a reguluje transkripci cílových genů (Mimura a Fujii-Kuriyama, 2003). Podávání 2,3,7,8-tetrachlorodibenzo-p-dioxinu (TCDD) potkanům zpomaluje transport cholesterolu do mitochondrie (Moore et al., 1991) a snižuje také aktivitu CYP17 (Mebus et al., 1987). Další studie v souladu s předchozími prokázala snížení exprese StAR, cytochromů P450 11A1, 17, 11B1 a 3βHSD ve varlatech mláďat potkanů, jejichž matky byly v graviditě vystaveny působení TCDD (Mutoh et al., 2006). Pro dioxiny je zajímavý poznatek, že expozice dioxinu TCDD v dětství snižuje koncentraci spermií a jejich motilitu, naopak, dojde-li k expozici během puberty, je vidět opačný účinek (Mocarelli et al., 2008).

V případě podávání xenoestrogenu bisfenolu A byla zjištěna snížená aktivita cytochromu CYP17 (Akingbemi et al., 2004) a stejně tak StAR, CYP11A1 a 17βHSD (Nakamura et al., 2010).

Působením na steroidogenní enzymy ED ovlivňují produkci testosteronu Leydigovými buňkami v průběhu fetálního vývoje i v dospělosti. Nejčastějším cílem ED jsou tedy geny kódující StAR, cytochrom P450scc a cytochrom P450 17a-hydroxylázu/17,20 lyázu, které jsou potřebné pro biosyntézu steroidních hormonů. Následkem změn ve steroidním spektru, které dále ovlivňují zpětnovazebné systémy na ose hypothalamus-hypofýza-gonády, dochází k reprodukční dysfunkci (Yeung et al., 2011).

Indukce oxidativního stresu

Stále více důkazů naznačuje, že chemikálie z prostředí mohou snižovat testikulární funkce také narušením rovnováhy mezi pro-oxidačními a antioxidačními mechanizmy, čímž aktivují další dráhy, např. dráhu vedoucí k apoptóze. Ačkoliv fyziologické hladiny reaktivních kyslíkových radikálů a apoptózy jsou nutné pro normální funkce varlat, patologické hladiny mohou být škodlivé (Mathur a D'Cruz, 2011).

Ve varlatech je vyvinuta řada antioxidačních mechanizmů, mezi nimiž působí jak enzymatické, tak neenzymatické složky (Aitken, Roman, 2008). Mezi antioxidační enzymy chránící varlata patří např. superoxid dismutáza, glutathion peroxidáza a kataláza. Superoxid dismutáza nejprve

konvertuje superoxidový anion na peroxid vodíku, který pak kataláza a superoxid dismutáza převede na vodu.

Kromě enzymů se na ochraně testes před oxidačním poškozením podílejí i molekuly s malou molekulovou hmotností, jakou je např. zinek, který je hlavní součástí antioxidačních enzymů, jako je superoxid dismutáza, chrání – SH skupiny v buňce před oxidací a snižuje lipidovou peroxidaci tak, že odstraňuje přechodné kovy, jako jsou železo a měď, z katalytických míst (Bray a Bettger, 1990). Mezi další antioxidanty s malou molekulovou hmotností patří vitamíny C a E. Nedostatky těchto vitamínů vedou ke stavům oxidativního stresu ve varlatech a tím k narušení spermatogeneze i steroidogeneze (Johnson, 1979). Zajímavou antioxidační molekulou je melatonin, který podstupuje 2-elektronovu oxidaci při jeho antioxidačním působení a navíc je rozpustný ve vodném i lipidovém prostředí, může proto snadno prostoupit hematotestikulární bariérou a chránit zárodečný epitel (Aitken a Roman, 2008).

Několik studií publikovalo výsledky ohledně bisfenolu A, ve kterých zvyšuje oxidační stres ve varlatech, nadvarlatech a spermiích u různých živočišných druhů. Podávání bisfenolu A potkanům způsobuje snížení motility i počtu spermií v epididymis, dále snižuje aktivitu superoxid dismutázy, katalázy, glutathion reduktázy a glutathion peroxidázy a s tím související zvýšení hladin peroxidu vodíku a lipidové peroxidace (Chitra et al., 2003). Ve varlatech byly u myší zjištěny snížené hodnoty redukovaného glutathionu po administraci bisfenolu A (Kabuto et al., 2003).

U endokrinních disruptorů, jako jsou např. bisfenol A, 2,3,7,8-tetrachlorodibenzo-p-dioxin či kadmium, je známo, že indukují oxidativní stres ve varlatech down-regulací antioxidačních enzymů (Dhanabalan a Mathur, 2009; Chitra et al., 2003; Patra et al., 2011). Nedávné studie ukázaly, že endokrinní disruptory mohou způsobit mužskou infertilitu také narušením buněčných spojení mezi Sertoliho a zárodečnou buňkou v testes a mezi Sertoliho buňkami navzájem. K takovému porušení integrity v testes může docházet buď přes signalizační dráhu mitogenně aktivované protein kinázy (MAPK), nebo přes kaskádu fosfatidylinositol-3-kinázy/c-Src/focal adhesion kinázy (Wong a Cheng, 2011). Narušení spojení mezi jednotlivými buňkami vede následně k dysregulaci spermatogeneze. Bisphenol A je jedním z disruptorů, které mohou působit přes MAPK dráhu a narušovat spojení „gap junction" mezi Sertoliho buňkami v hematotestikulární bariéře (Cheng et al., 2011).

Epigenetické působení ED

Všechny doposud jmenované mechanizmy vysvětlují přímé působení ED na organizmus. Relativně nově bylo zjištěno, že vývoj zárodečných buněk může být ovlivněn také nepřímo, zděděným epigenetickým působením ED. Epigenetické změny jsou takové změny v genové expresi, při kterých DNA sekvence zůstává zachována, ale upravena může být např. metylace DNA, acetylace histonů či microRNA (Baccarelli a Bollati, 2009). Dostupné informace z modelů na zvířatech ukazují, že při expozici xenobiotikům v kritických obdobích savčího vývoje může docházet k trvalým a dědičným změnám v epigenetickém stavu. Poprvé byly epigenetické změny v zárodečné linii prezentovány u potkanů Anwayem a kol. (2005). Březí samice potkanů byly vystaveny antiandrogennímu fungicidu vinclozolinu či estrogennímu insekticidu methoxychloru. V F1 generaci byl pozorován snížený počet a viabilita spermií. Tyto účinky byly pozorovány ve všech zkoumaných generacích (až do F4) a korelovaly se změnami v metylaci DNA (Anway et al., 2005). Další studie prokázaly hypometylaci DNA při prenatální expozici BPA u potkanů (Dolinoy et al., 2007) či negativní korelaci mezi hypometylací DNA a hladinami perzistentních organických polutantů (např. DDT, p,p´-DDE) v séru (Rusiecki et al., 2008).

Závěr

Během života jsou lidé i zvířata vystaveni množství chemikálií, z nichž významný počet může zasahovat do endokrinního systému organizmu. U některých látek již jsou nepříznivé účinky dokázány, u jiných se čeká na jejich odhalení. Tyto účinky jsou prozkoumány hlavně na zvířatech a epidemiologických datech. Zdá se býti velmi pravděpodobné, že ED působí i na mužský reprodukční systém. Mechanizmy jejich účinků mohou být různé, ale mají společný následek, zhoršené fertilizační schopnosti. Stále v této oblasti existuje omezené množství studií, z nichž jich řada ukazuje na protichůdné výsledky. Proto je potřeba dalších výzkumů k prokázání přesných vztahů mezi ED a mužským reprodukčním systémem. Důležité je mimo jiné prozkoumat mechanizmy působení více ED najednou, protože organizmus je v drtivé většině vystaven směsi ED, která mohou působit aditivně či dokonce synergisticky. Neméně závažné je působení disruptorů během vývoje organizmu, kdy mohou být následky časově odložené od expozice a patrné až v dospělosti. I poznatky o mechanizmech působení ED jsou stále neúplné.

Poděkování: Přehled vznikl za podpory projektu IGA MZ ČR NT 13369–4 a MZ ČR – RVO (Endokrinologický ústav – EÚ, 00023761).

LITERATURA

1. Adami HO, Bergstrom R, Mohner M, Zatonski W, Storm H, Ekbom A, Tretli S, Teppo L, Ziegler H, Rahu M et al. Testicular cancer in nine northern European countries. Int J Cancer 1994; 59: 33-8.
2. Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev 2008; 1: 15-24.
3. Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP. Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology 2004; 145: 592-603.
4. Akingbemi BT, Youker RT, Sottas CM, Ge R, Katz E, Klinefelter GR, Zirkin BR, Hardy MP. Modulation of rat Leydig cell steroidogenic function by di(2-ethylhexyl)phthalate. Biol Reprod 2001; 65: 1252-9.
5. Andersson AM, Jensen TK, Juul A, Petersen JH, Jorgensen T, Skakkebaek NE. Secular decline in male testosterone and sex hormone binding globulin serum levels in Danish population surveys. J Clin Endocrinol Metab 2007; 92: 4696-705.

6. Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 2005; 308: 1466-9.
7. Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM, Strauss JF, 3rd. Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. J Biol Chem 1997; 272: 32656-62.
8. Arnold SF, Klotz DM, Collins BM, Vonier PM, Guillette LJ, Jr., McLachlan JA. Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science 1996; 272: 1489-92.
9. Auger J, Kunstmann JM, Czyglik F, Jouannet P. Decline in semen quality among fertile men in Paris during the past 20 years. N Engl J Med 1995; 332: 281-5.
10. Baccarelli A, Bollati V. Epigenetics and environmental chemicals. Curr Opin Pediatr 2009; 21: 243-51.
11. Barker DJ. The developmental origins of adult disease. Eur J Epidemiol 2003; 18: 733-6.
12. Bray TM, Bettger WJ. The physiological role of zinc as an antioxidant. Free Radic Biol Med 1990; 8: 281-91.
13. Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. BMJ 1992; 305: 14. de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl 2000; 21: 776-98.
15. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci U S A 2003; 100: 10972-6.
16. Dhanabalan S, Mathur PP. Low dose of 2,3,7,8 tetrachlorodibenzo-p-dioxin induces testicular oxidative stress in adult rats under the influence of corticosterone. Exp Toxicol Pathol 2009; 61: 415-23.
17. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev 2009; 30: 293-342.
18. Dolinoy DC, Huang D, Jirtle RL. Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. Proc Natl Acad Sci U S A 2007; 104: 13056-61.
19. Dufau ML. The luteinizing hormone receptor. Annu Rev Physiol 1998; 60: 461-96.
20. Feldman HA, Longcope C, Derby CA, Johannes CB, Araujo AB, Coviello AD, Bremner WJ, McKinlay JB. Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts male aging study. J Clin Endocrinol Metab 2002; 87: 589-98.
21. Fisch H, Goluboff ET, Olson JH, Feldshuh J, Broder SJ, Barad DH. Semen analyses in 1,283 men from the United States over a 25-year period: no decline in quality. Fertil Steril 1996; 65: 1009-14.
22. Griswold MD. The central role of Sertoli cells in spermatogenesis. Semin Cell Dev Biol 1998; 9: 411-6.
23. Hauet T, Liu J, Li H, Gazouli M, Culty M, Papadopoulos V. PBR, StAR, and PKA: partners in cholesterol transport in steroidogenic cells. Endocr Res 2002; 28: 395-401.
24. Cheng CY, Wong EW, Lie PP, Li MW, Su L, Siu ER, Yan HH, Mannu J, Mathur PP, Bonanomi M, Silvestrini B, Mruk DD. Environmental toxicants and male reproductive function. Spermatogenesis 2011; 1: 2-13.
25. Chilvers C, Pike MC, Forman D, Fogelman K, Wadsworth ME. Apparent doubling of frequency of undescended testis in England and Wales in 1962-81. Lancet 1984; 2: 330-2.
26. Chitra KC, Latchoumycandane C, Mathur PP. Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology 2003; 185: 119-27.
27. Irvine S, Cawood E, Richardson D, MacDonald E, Aitken J. Evidence of deteriorating semen quality in the United Kingdom: birth cohort study in 577 men in Scotland over 11 years. BMJ 1996; 312: 467-71.
28. Johnson FC. The antioxidant vitamins. CRC Crit Rev Food Sci Nutr 1979; 11: 217-309.
29. Kabuto H, Hasuike S, Minagawa N, Shishibori T. Effects of bisphenol A on the metabolisms of active oxygen species in mouse tissues. Environ Res 2003; 93: 31-5.
30. Lagos-Cabre R, Moreno RD. Contribution of environmental pollutants to male infertily: a working model of germ cell apoptosis induced by plasticizers. Biol Res 2012; 45: 5-14.
31. Mathur PP, D'Cruz SC. The effect of environmental contaminants on testicular function. Asian J Androl 2011; 13: 585-91.
32. Mebus CA, Reddy VR, Piper WN. Depression of rat testicular 17-hydroxylase and 17,20-lyase after administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Biochem Pharmacol 1987; 36: 727-31.
33. Mimura J, Fujii-Kuriyama Y. Functional role of AhR in the expression of toxic effects by TCDD. Biochim Biophys Acta 2003; 1619: 263-8.
34. Mocarelli P, Gerthoux PM, Patterson DG, Jr., Milani S, Limonta G, Bertona M, Signorini S, Tramacere P, Colombo L, Crespi C, Brambilla P, Sarto C, Carreri V, Sampson EJ, Turner WE, Needham LL. Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. Environ Health Perspect 2008; 116: 70-7.
35. Moore RW, Jefcoate CR, Peterson RE. 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibits steroidogenesis in the rat testis by inhibiting the mobilization of cholesterol to cytochrome P450scc. Toxicol Appl Pharmacol 1991; 109: 85-97.
36. Mutoh J, Taketoh J, Okamura K, Kagawa T, Ishida T, Ishii Y, Yamada H. Fetal pituitary gonadotropin as an initial target of dioxin in its impairment of cholesterol transportation and steroidogenesis in rats. Endocrinology 2006; 147: 927-36.
37. Nakamura D, Yanagiba Y, Duan Z, Ito Y, Okamura A, Asaeda N, Tagawa Y, Li C, Taya K, Zhang SY, Naito H, Ramdhan DH, Kamijima M, Nakajima T. Bisphenol A may cause testosterone reduction by adversely affecting both testis and pituitary systems similar to estradiol. Toxicol Lett 2010; 194: 16-25.
38. O'Donnell L, Robertson KM, Jones ME, Simpson ER. Estrogen and spermatogenesis. Endocr Rev 2001; 22: 289-318.
39. Papadopoulos V. In search of the function of the peripheral-type benzodiazepine receptor. Endocr Res 2004; 30: 677-84.
40. Patra RC, Rautray AK, Swarup D. Oxidative stress in lead and cadmium toxicity and its amelioration. Vet Med Int 2011; 2011: 457327.
41. Paulozzi LJ. International trends in rates of hypospadias and cryptorchidism. Environ Health Perspect 1999; 107: 297-302.
42. Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 2004; 25: 947-70.
43. Phillips KP, Tanphaichitr N. Human exposure to endocrine disrupters and semen quality. J Toxicol Environ Health B Crit Rev 2008; 11: 188-220.

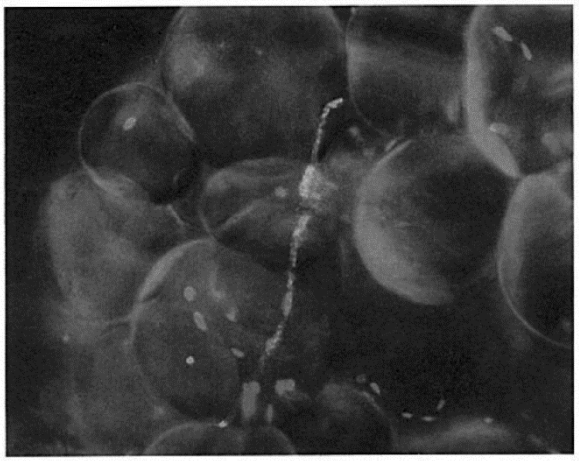
44. Rance NE. Menopause and the human hypothalamus: evidence for the role of kisspeptin/neurokinin B neurons in the regulation of estrogen negative feedback. Peptides 2009; 30: 111-22.
45. Roseweir AK, Millar RP. The role of kisspeptin in the control of gonadotrophin secretion. Hum Reprod Update 2009; 15: 203-12.
46. Rusiecki JA, Baccarelli A, Bollati V, Tarantini L, Moore LE, Bonefeld-Jorgensen EC. Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect 2008; 116: 1547-52.
47. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF, Jr., Aparicio SA, Colledge WH. The GPR54 gene as a regulator of puberty. N Engl J Med 2003; 349: 1614-27.
48. Seo JT, Rha KH, Park YS, Lee MS. Semen quality over a 10-year period in 22,249 men in Korea. Int J Androl 2000; 23: 194-8.
49. Sharma H, Zhang P, Barber DS, Liu B. Organochlorine pesticides dieldrin and lindane induce cooperative toxicity in dopaminergic neurons: role of oxidative stress. Neurotoxicology 2010; 31: 215-22.
50. Sharpe RM. Environmental/lifestyle effects on spermatogenesis. Philos Trans R Soc Lond B Biol Sci 2010; 365: 1697-712.
51. Schug TT, Janesick A, Blumberg B, Heindel JJ. Endocrine disrupting chemicals and disease susceptibility. J Steroid Biochem Mol Biol 2011; 127: 204-15.
52. Silveira LF, Teles MG, Trarbach EB, Latronico AC. Role of kisspeptin/GPR54 system in human reproductive axis. Front Horm Res 2010; 39: 13-24.
53. Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 2005; 146: 3686-92.
54. Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol 2001; 63: 193-213.
55. Swan SH, Elkin EP, Fenster L. Have sperm densities declined? A reanalysis of global trend data. Environ Health Perspect 1997; 105: 1228-32.
56. Wade MG, Desaulniers D, Leingartner K, Foster WG. Interactions between endosulfan and dieldrin on estrogen-mediated processes in vitro and in vivo. Reprod Toxicol 1997; 11: 791-8.
57. Walker WH, Cheng J. FSH and testosterone signaling in Sertoli cells. Reproduction 2005; 130: 15-28.
58. Wang RS, Yeh S, Tzeng CR, Chang C. Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocr Rev 2009; 30: 119-32.
59. Watanabe H, Suzuki A, Kobayashi M, Lubahn DB, Handa H, Iguchi T. Similarities and differences in uterine gene expression patterns caused by treatment with physiological and non-physiological estrogens. J Mol Endocrinol 2003; 31: 487-97.
60. Wong EW, Cheng CY. Impacts of environmental toxicants on male reproductive dysfunction. Trends Pharmacol Sci 2011; 32: 290-9.
61. Yeung BH, Wan HT, Law AY, Wong CK. Endocrine disrupting chemicals: Multiple effects on testicular signaling and spermatogenesis. Spermatogenesis 2011; 1: 231-239.
62. Zvěřina J, Urbánek V, Cirýn, J. Spermiologické parametry českých mužů v letech 1950 – 1984. Sborník lékařský 2002; 103(1): 35-47.

Mgr. Jana Kubátová
Endokrinologický ústav
Národní 8
116 94 Praha 1
e-mail: jkubatova@endo.cz

PŘÍLOHA IV

Kubátová J, Stárka L, Bičíková M, Hampl R: Endokrinní disruptory – sílící hrozba pro lidskou populaci i volně žijící organismy. Lékařské listy, 11, 14 – 16 (2013).

ENDOKRINOLOGIE



Ilustrační foto: newswise.com

žení viscerální tukové tkáně o 66–129 %, přičemž prevalence obezity infikovaných opic dosáhla 100 %. Studie provedené na vzorcích lidské tukové tkáně infikovaných virem AD-36 prokázaly zmnožení tukových buněk a zvýšenou expresi některých enzymů, jako jsou např. lipoproteinová lipáza a syntáza mastných kyselin.

Primárním místem působení tohoto infekčního agens je tuková tkáň, kde se prostřednictvím bílkoviny označované jako E4orf1 aktivují enzymy účastnící se adipogeneze. Tato bílkovina je navíc zodpovědná za zvýšené vychytávání glukózy v tukové tkáni nezávisle na inzulinové signalizaci a současně za snížené uvolňování glukózy z jater. Podobně jako u dospělých prokázaly i studie u dětí a adolescentů 2–3krát vyšší prevalenci pozitivity protilátek proti AD-36 u obézních jedinců oproti jedincům s normální tělesnou hmotností.

Studie COPAT

Studie Childhood Obesity Prevalence And Treatment (COPAT), realizovaná Endokrinologickým ústavem, popisuje mimo jiné souvislost pozitivity protilátek proti AD-36 s obezitou a metabolickými riziky u 1179 jedinců, a představuje tak doposud největší takto vyšetřený soubor adolescentů. Pozitivita protilátek proti AD-36 významně souvisela s tělesnou hmotností (p = 0,042), BMI (p = 0,015), obvodem boků (p = 0,004), Z-skóre tělesné výšky (p = 0,029), celkovým tělesným tukem (p = 0,000) a s tukem na trupu (p = 0,000). U adolescentů vykazujících pozitivitu AD-36 byly zjištěny vyšší koncentrace celkového a LDL cholesterolu při signifikantně nižších glykemiích nalačno. Vztah infekce AD-36 ke koncentraci adiponektinu ale zaznamenán nebyl.

Je otázkou, zda infekce AD-36 nemůže souviset s „metabolicky zdravou obezitou" a zda se protein E4orf1 nemůže stát základem pro vývoj nových antidiabetik, která by vedle diabetu mohla příznivě ovlivňovat nealkoholickou steatózu jater a lipodystrofii.

Podpořeno grantem z Norska prostřednictvím Norského finančního mechanismu CZ 0123), grantem 7F08077 z MSM/7F a projektem MZ ČR – RVO (Endokrinologický ústav – EÚ, 00023761).

RNDr. Hana Zamrazilová, Ph.D.,
MUDr. Irena Aldhoon Hainerová, Ph.D.,
doc. MUDr. Vojtěch Hainer, CSc.
Centrum pro diagnostiku a léčbu obezity, Endokrinologický ústav, Praha

Endokrinní disruptory – sílící hrozba pro lidskou populaci i volně žijící organismy

V posledních dekádách se objevuje stále více důkazů o zvýšeném výskytu různých hormonálních poruch u lidí i volně žijících zvířat. Prevalence obezity, diabetes mellitus 2. typu, nádorů spojených s endokrinním systémem, jako jsou karcinomy prsu, endometria, ovarií, prostaty, varlat i štítné žlázy stoupá, naopak klesá kvalita spermií a celková plodnost mužů i žen. Tyto skutečnosti jsou dávány do souvislosti se zvyšujícím se výskytem antropogenních látek v prostředí, které mají účinky na endokrinní systém. Takové látky nazýváme endokrinní disruptory.

Ilustrační foto: 123.co.uk

Již v roce 1992 byla publikována rozsáhlá metaanalýza, kde bylo prezentováno postupné snižování počtu spermií u mužů z různých částí světa v průběhu uplynulých padesáti let. Existují zde sice geografické odlišnosti, ale je jisté, že např. 20–40 % mladých mužů v Dánsku, Finsku, Německu, Norsku či Švédsku mají počty spermií v subfertilních mezích. U žen jsou diskutovány účinky disruptorů ve vztahu k mimoděložním těhotenstvím, předčasným porodům, potratům i neplodnosti. Známý je příklad diethylstilbestrolu jakožto syntetického estrogenu, který byl předepisován v letech 1938–1971 těhotným ženám ve Spojených státech amerických jako prevence proti potratům a předčasným porodům. U dcer těchto matek byla později častěji diagnostikována vzácná forma vaginálního adenokarcinomu a u 90–95 % z nich byly pozorovány benigní reprodukční problémy.

Endokrinní disruptory jsou chemicky různorodá skupina látek,

které se nacházejí v prostředí všude kolem nás. Obsaženy jsou zejména v potravinách, plastových láhvích a dalších obalech či v elektronice, hračkách a kosmetických přípravcích, pesticidech a fungicidech. Do organismu se dostanou zejména požitím kontaminované potravy, vypitím kontaminované vody, dýcháním znečištěného vzduchu či transdermálně. Účinky disruptorů jsou také často popisovány na případech náhodné masivní expozice v zaměstnání.

Mechanismy působení endokrinních disruptorů

Původně se soudilo, že endokrinní disruptory působí především přes jaderné receptory, jako jsou estrogenní, androgenní, progestinové, thyroidní receptory či receptory retinoidní. Disruptor se díky vlastnostem podobným endogenním hormonům naváže na jaderný receptor, celý komplex se následně přesune k DNA, kde se naváže na hormon-responzivní element a zde působí jako transkripční faktor, který spouští kaskádu dějů vedoucích k transkripci cílových genů. Pokusy s estradiolem a diethylstilbestrolem ukázaly, že diethylstilbestrol se sice naváže na estrogenní receptor stejně jako estradiol, ale některé geny se exprimují více a jiné naopak méně, takže výsledný efekt těchto dvou látek je rozdílný. Jsou známy i další receptory, jako membránový steroidní receptor pro estrogeny, receptory pro arylované uhlovodíky, receptory aktivované proliferátory peroxisomů γ (PPARγ) a další nesteroidní receptory, přes které mohou disruptory působit.

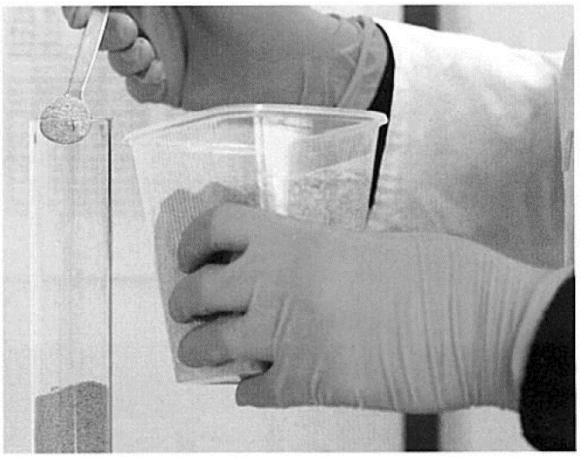
Dnes již víme, že endokrinní disruptory působí nejen přes receptory, ale mohou působit i na jiných úrovních regulace hormonů, jako je degradace, transport a biosyntéza hormonů. Ovlivněním enzymových systémů dojde k narušení křehké rovnováhy mezi regulačními mechanismy v endokrinním systému, které pak mají zásadní vliv na koncentrace hormonů v organismu. V případě bisfenolu A, známého disruptoru, který se nachází v plastových láhvích, a který se následně uvolňuje do jejich obsahu, byla zjištěna snížená aktivita steroidního akutního regulačního proteinu (StAR), kontrolující transport cholesterolu do mitochondrií a enzymů zahrnutých do steroidní biosyntézy a metabolismu jako cholesterol desmoláza, 17α-hydroxyláza/17,20lyáza, 17β-hydroxysteroidní dehydrogenáza i 11β-hydroxysteroidní dehydrogenáza. Následkem změn ve steroidním spektru, které dále ovlivňují zpětnovazebné systémy na osách řízených hypothalamem, dochází např. k reprodukční dysfunkci. Disruptory nepůsobí jen na enzymy ve steroidní cestě, ale mohou downregulovat i antioxidační enzymy, které se starají o odstraňování škodlivých kyslíkových radikálů v buňce. Mezi antioxidační enzymy chránící buňku patří např. superoxid dismutáza, glutathion peroxidáza a kataláza.

Všechny doposud jmenované mechanismy vysvětlují přímé působení ED na organismus. Relativně nedávno bylo zjištěno, že vývoj zárodečných buněk může být ovlivněn také nepřímo, zděděným epigenetickým působením ED. Epigenetické změny jsou takové změny v genové expresi, při kterých DNA sekvence zůstává zachována, ale upravená může být např. metylace DNA, acetylace histonů či microRNA. Dostupné informace z modelů na zvířatech ukazují, že při expozici xenobiotikům v kritických obdobích savčího vývoje může docházet k trvalým a dědičným změnám v epigenetickém stavu.

Zvláštnosti působení disruptorů

Endokrinní disruptory svými účinky vykazují některé zajímavé charakteristiky. Paradoxně působí nepříznivěji v extrémně nízkých koncentracích než ve větších dávkách. V tomto se podobají endogenním hormonům, které mimikují a které také působí v nanomolárních koncentracích.

Zásadní v případě disruptorů je věk v době expozice. Mohou sice



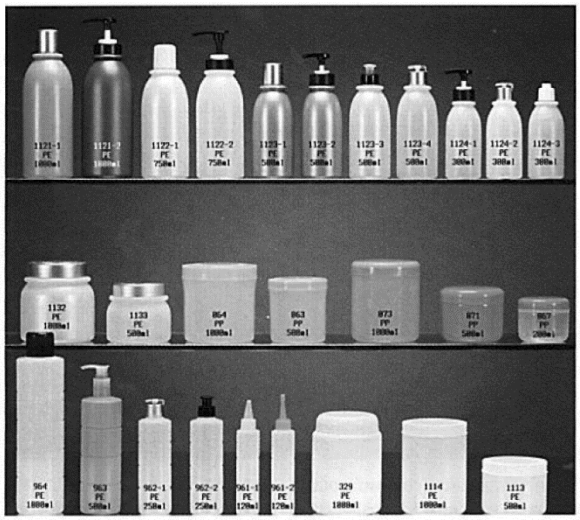
Ilustrační foto: politiken.dk

působit v průběhu celého života, ale vystavení se ED v dospělosti může mít zcela odlišné důsledky než expozice během vývoje. U dospělých je zpravidla potřeba vyšší hladina ED, aby působila toxicky na organismus. Naopak během vývoje organismu stačí nízká dávka po kratší dobu a může mít trvalé následky až do dospělosti, kdy už endokrinní disruptory dávno v těle nejsou přítomny. Tento koncept je pojmenován „the fetal basis of adult disease" neboli expozice ED při vývoji organismu je základem nemoci/poruchy v dospělosti. Důvody pro vysokou citlivost k disruptorům ve fetálním a raně postnatálním období jsou funkční a strukturální změny, které v tu dobu probíhají. Svou roli v této fázi vývoje mají pohlavní hormony v diferenciaci pohlaví a thyroidní hormony ve vývoji mozku, se kterými mohou disruptory interagovat. Na rozdíl od dospělého organismu zde také ještě nejsou plně rozvinuty reparační mechanismy k ochraně DNA, imunitní systém, detoxikační enzymy, jaterní metabolismus a hematoencefalická bariéra. Mezi další kritická období v životě jedince patří dětství a puberta.

V prostředí existuje velké množství látek, které mohou narušovat endokrinní rovnováhu a organismus proto není vystaven působení jen jedné chemikálie, ale spíše koktejlu různých látek. Tyto látky pak mohou v těle organismu vykazovat aditivní nebo dokonce synergické účinky, kdy efekt společného působení není jen prostý součet působení látek, ale je obvykle větší nebo kvalitativně lepší. Mnohé z disruptorů jsou velmi perzistentní (PCB, dioxiny), což má za následek jejich bioakumulaci v potravním řetězci a také v lidském organismu, jiné se naopak rychle rozkládají, a mohou tak působit jen po omezenou dobu. Tyto skutečnosti pak velice ztěžují zjišťování negativních účinků disruptorů in vivo.

Nedávno byla také publikována studie o dalších neprozkoumaných vlastnostech některých syntetických hormonálních látek v prostředí. Ve Spojených státech amerických je používán trenbolon acetát jako růstový preparát pro hovězí dobytek. V těle dobytku se tento anabolický steroid rozkládá na 17α-trenbolon, 17β-trenbolon a trendion. O prvých dvou metabolitech je známo, že to jsou účinné endokrinní disruptory. Mělo se za to, že po opuštění těla zvířete tyto látky degradují v prostředí a nemají pak vliv na ostatní organismy. Opak se ukázal pravdou, bylo prokázáno, že za denního světla tyto látky sice degradují na neaktivní metabolity, ovšem tato reakce je reverzibilní a během noci se až 60 % inaktivních metabolitů změní zpět na účinné endokrinní disruptory. Uvedený výsledek v této problematice je velice zásadní

ENDOKRINOLOGIE



Ilustrační foto: nhfengmei-en.alibaba.com

a je možné, že i další endokrinní disruptory jsou schopné této reverzibilní fotoreakce. Bylo to prokázáno už v případě dienogestu, což je progestin používaný v orální antikoncepci, či u diendionu, anabolického steroidu ilegálně používaného kulturisty k růstu svalové hmoty.

Obezogeny – speciální skupina endokrinních disruptorů

Role hormonů a potažmo i endokrinních disruptorů v metabolických poruchách jako obezita a diabetes, je v poslední době velmi diskutovaná. Důvody, proč tomu tak je, vycházejí ze studií na zvířatech. Ty naznačují, že vystavení se určitým endokrinním disruptorům během kritických období ve vývoji organismu může vést k narušenému lipidovému metabolismu, přibývání na váze a vzniku diabetu druhého typu v pozdějších fázích života. Poznatky u lidské populace jsou omezené, nicméně faktem je, že kouření během těhotenství vede k nárůstu váhy potomka později v dospělosti.

Jako obezogeny jsou označovány chemikálie, které podporují obezitu zvyšováním počtu tukových buněk (a zvyšováním obsahu tuku v již existujících buňkách), změnou počtu kalorií spáleném při klidovém metabolismu a zasahují do regulačních mechanismů, které ovlivňují chuť k jídlu a sytost. Typickým a nejdéle známým obezogenem je tributyltin. Váže se v nanomolárních koncentracích jak na PPARγ, tak na jeho heterodimerního partnera, retinoidní X receptor, a indukuje adipogenezi. Tributyltin se využívá jako fungicid nebo do přípravků k impregnaci dřeva. Studie s tímto endokrinním disruptorem na myších ukázaly, že expozice tributyltinu in utero vedla k předčasnému ukládání tuku v tukové tkáni potomka a tato tendence k uchovávání tuku byla přeprogramovaná ještě před porodem.

Nadměrná konzumace nezdravých jídel a nedostatečná pohybová aktivita bezpochyby vedou k obezitě. Zda jsou tyto dvě příčiny těmi nejdůležitějšími ve vzniku obezity, nebo zda jsou tu další významné faktory, které k ní přispívají, zatím není jasné. Přibývá ovšem důkazů o významu endokrinních disruptorů v programování obezity v raném věku. Do budoucna bude potřeba zjistit, kolik takových látek kauzálně spojených s obezitou je.

Legislativa endokrinních disruptorů

Pojem endokrinní disruptor poprvé zazněl na konferenci vědců ve Wingspreadu ve státě Wisconsin v roce 1991, kde zaznělo, že „mnoho látek uvedených do prostředí lidskou činností je schopných narušovat endokrinní systém zvířat, včetně ryb, volně žijících živočichů a člověka". Od té doby se začala řešit problematika ED a jejich potenciální nepříznivé účinky na člověka.

Většina zákonů o environmentálních kontaminantech ve Spojených státech amerických je připravována US Environmental Protection Agency (USEPA), která identifikovala asi 87 000 chemikálií, které jsou komerčně využívány. O drtivé většině z těchto látek máme jen málo informací o jejich toxicitě. Proto v roce 1998 USEPA navrhla 2pilířový postup na testování toxicity chemikálií. První pilíř zahrnuje screeningovou baterii in vitro a in vivo testů navrženou pro testování potenciálních interakcí látek s endokrinním systémem. Ty látky, které budou vyhodnoceny jako potenciálně interagující, postoupí do druhého pilíře pro určení nežádoucích účinků na organismus a určení dynamiky dávky a odpovědi. Výsledky testů z druhého pilíře pak poslouží k posouzení nebezpečnosti látek, které povedou k regulaci těchto látek na trhu.

V Evropské unii je hlavním regulačním orgánem ECHA (European Chemical Agency), která se stará o tvorbu a uplatňování právních předpisů o chemických látkách, které vedou k ochraně lidského zdraví a prostředí. V roce 2007 vstoupila v platnost směrnice REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals). Ta zjednodušuje a zlepšuje dřívější legislativní rámec pro chemické látky v Evropské unii a činí průmysl odpovědným za stanovení a vyhodnocení rizik chemikálií a uživatelům musí poskytnout informace o látkách, které vzbuzují mimořádné obavy. Na kandidátské listině látek vzbuzujících mimořádné obavy figuruje v současnosti 144 chemikálií. V květnu letošního roku skončila druhá vlna registrace chemikálií, které jsou dovážené jednou společností do Evropské unie v množstvích 100 až 1000 tun ručně. Poslední vlna registrace má proběhnout do konce května 2018, kdy mají být zaregistrovány chemikálie dovážené do EU v množství menší, než 1 tuna ročně.

Téma endokrinních disruptorů je v poslední době velmi diskutované. V červnu letošního roku 89 předních vědců z celého světa sepsalo Berlaymontskou deklaraci o endokrinních disruptorech. V té vyjádřili svůj názor k tomuto důležitému tématu a vyzvali Evropskou komisi k zavedení regulačních opatření, která jsou v souladu se současnými nejnovějšími vědeckými poznatky a metodami. Podobné stanovisko zaujímá Evropský parlament, který jednomyslně schválil zprávu europoslankyně Åsy Westlund o ochraně veřejného zdraví před endokrinními disruptory. Ta vyzývá k přijetí opatření, která by byla zaměřena na snížení krátkodobé a dlouhodobé expozice osob endokrinním disruptorům. Navrhuje také daleko více se zaměřit na výzkum, který by zlepšil vědecké poznatky o vlivu látek s negativním působením na endokrinní systém a lidské zdraví.

Do budoucna je potřeba lépe prozkoumat rizika endokrinních disruptorů, aby se snížila expozice endokrinních disruptorů během vývoje organismu a mohlo se předcházet rizikům disruptory vyvolaných onemocnění. Výzvou pro další výzkum je i vývoj analytických metod pro jejich identifikaci a screening, které by odpovídaly požadavkům na legislativu. V neposlední řadě pak je zde snaha o porozumění účinkům směsí ED na organismus a poznání mechanismů, které za tím stojí.

Studiu endokrinních disruptorů se můžeme věnovat díky podpoře IGA MZ ČR NT 13369-4 a projektu 00023761 MZ ČR koncepčního rozvoje výzkumu EU.

Mgr. Jana Kubátová,
prof. MUDr. RNDr. Luboslav Stárka, DrSc.,
RNDr. Marie Bičíková,
prof. RNDr. Richard Hampl, DrSc.,
Endokrinologický ústav, Praha

PŘÍLOHA V

Hampl R, Kubátová J, Stárka L: Steroids and endocrine disruptors – History, recent state of art and open questions, J Steroid Biochem Mol Biol (2014) In press, available online. IF = 3.628

Journal of Steroid Biochemistry & Molecular Biology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

Steroids and endocrine disruptors—History, recent state of art and open questions

Richard Hampl*, Jana Kubátová, Luboslav Stárka

Institute of Endocrinology, Národní 8, 116 94 Praha 1, Czech Republic

ARTICLE INFO

Article history:
Received 24 March 2014
Received in revised form 14 April 2014
Accepted 20 April 2014
Available online xxx

Keywords:
Steroids
Endocrine disruptors
Sites of action
Overreview

ABSTRACT

This introductory chapter provides an overview of the levels and sites at which endocrine disruptors (EDs) affect steroid actions. In contrast to the special issue of Journal of Steroid Biochemistry and Molecular Biology published three years ago and devoted to EDs as such, this paper focuses on steroids. We tried to point to more recent findings and opened questions.

EDs interfere with steroid biosynthesis and metabolism either as inhibitors of relevant enzymes, or at the level of their expression. Particular attention was paid to enzymes metabolizing steroid hormones to biologically active products in target cells, such as aromatase, 5α-reductase and 3β-, 11β- and 17β-hydroxysteroid dehydrogenases. An important target for EDs is also steroid acute regulatory protein (StAR), responsible for steroid precursor trafficking to mitochondria.

EDs influence receptor-mediated steroid actions at both genomic and non-genomic levels. The remarkable differences in response to various steroid-receptor ligands led to a more detailed investigation of events following steroid/disruptor binding to the receptors and to the mapping of the signaling cascades and nuclear factors involved. A virtual screening of a large array of EDs with steroid receptors, known as in silico methods (=computer simulation), is another promising approach for studying quantitative structure activity relationships and docking.

New data may be expected on the effect of EDs on steroid hormone binding to selective plasma transport proteins, namely transcortin and sex hormone-binding globulin.

Little information is available so far on the effects of EDs on the major hypothalamo–pituitary–adrenal/gonadal axes, of which the kisspeptin/GPR54 system is of particular importance. Kisspeptins act as stimulators for hormone-induced gonadotropin secretion and their expression is regulated by sex steroids via a feed-back mechanism. Kisspeptin is now believed to be one of the key factors triggering puberty in mammals, and various EDs affect its expression and function.

Finally, advances in analytics of EDs, especially those persisting in the environment, in various body fluids (plasma, urine, seminal fluid, and follicular fluid) are mentioned. Surprisingly, relatively scarce information is available on the simultaneous determination of EDs and steroids in the same biological material.

This article is part of a Special Issue entitled 'Endocrine disruptors & steroids'.

Contents

Abbreviations: BPA, bisphenol A; DDE, 2,2′-bis-(4-chlorophenyl)-1,1′-dichloroethene; DDT, dichlorodiphenyltrichloroethane; FSH, folitropin; LH, lutropin; PCB(s), polychlorinated biphenyl(s); PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; SHBG, sex hormone-binding globulin.

* Corresponding author. Tel.: +420 224905289; fax: +420 224905412.
E-mail addresses: rhampl@endo.cz, richardhampl@email.cz (R. Hampl).

http://dx.doi.org/10.1016/j.jsbmb.2014.04.013
0960-0760/

Please cite this article in press as: R. Hampl, et al., Steroids and endocrine disruptors—History, recent state of art and open questions, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.04.013

1. Introduction

1.1. History

The term endocrine disruptors (EDs) as substances which can interfere with the endocrine or hormone system in mammals dates back to the early nineties [1], but it was well known much earlier that many chemicals present in the environment may affect, mostly adversely, human and animal life and health. This includes many civilization diseases across the life cycle, such as cancer, genetic modification, metabolic diseases, the malfunction of various organs and, last but not least, reproduction. Concerning their effect on reproduction and, in broader terms the endocrine system, it is not surprising that a great part of this matter deals with steroids. This has been the subject of numerous reviews and monographs, such as that of Gore [2]. Some of these topics were addressed and discussed in this journal two years ago [3].

1.2. Classification

EDs may be divided into natural compounds such as soy phytoestrogens, extracts and formulas from various plants or fungi and a broad spectrum of human and industrial products. The latter comprise chemicals used in fighting undesired wildlife and agricultural threats (pesticides, fungicides, insecticides, and rodenticides) or various synthetic compounds as substances used in the production of plastics and plasticizers, packaging materials, including also non-intentionally added substances (bisphenol(s), phthalates) or a broad spectrum of industrial chemicals used as building materials, paints, isolation materials (PCBs, metals). Endocrine disruptors also include many drugs derived from natural hormones, particularly all contraceptives. Given their duration in the environment, EDs are usually divided into persistent and short-life compounds. The use of many of these chemicals (see e.g. DDT) was banned after their effects on wild life were discovered, but they still persist in the environment.

1.3. Effects

Generally, EDs may intervene in the hormonal function at various sites: they can directly affect hormone biosynthesis, the metabolism, transport, and mechanism of action on both receptor and post-receptor levels. They may act at a genome level by influencing gene expression and even via epigenetic mechanisms, including effects on genomic imprinting. Many EDs are known to affect fetal (prenatal) development. Other characteristic features of some EDs are their transgenerational effects and their often non-linear or non-monotonic dose–response curves. Some EDs act in an additive way with natural hormones and act complexly upon multiple targets. Finally, EDs can interfere with feed-back mechanisms typical for the endocrine system.

In the following text we will not repeat known facts about EDs, but focus on their effect on steroids, namely their biosynthesis, metabolism, mechanism of action, their co-existence with steroids in body fluids as potential biomarkers, and also discuss their participation in feed-back mechanisms in an attempt to point to open questions.

2. EDs and steroid biosynthesis and metabolism

EDs may influence steroid biosynthesis and metabolism either as inhibitors or rarely as activators of key enzymes, or on the level of the respective enzyme expression. Many excellent reviews have appeared since the beginning of this century demonstrating *in vitro* as well as in vivo inhibitory effects of an array of EDs, covering most of their classes – pesticides, plasticizers, dioxins, PCBs and polycyclic aromatic hydrocarbons, and their impact on ovarian or testicular functions [4–9]. The reviews covered all steroidogenic enzyme issues, most of which belong to the cytochrome P450 family [4,5,7]. Some of them dealt preferably with gonadal, ovarian [7,8] or Leydig cell [6,9] steroid biosynthesis.

Particular attention was devoted to aromatase activity [8], not only in humans or rodents, but also in fish regarding the strong impact of EDs pollutants in water on fish reproduction [10]. In rodents, it was shown that bisphenol A from plasticizers may even increase aromatase activity in rat prostate. At the same time, this chemical affects the expression of another important steroid metabolizing enzyme – 5α-reductase – existing in form of three isoenzymes (5α-R1, 5α-R2 and 5α-R3). While the expression of the first two isoenzymes is inhibited by bisphenol A, the third, 5α-R3, known as a biomarker for malignancy, is increased. It is an example of the synergic effect of the disruptor at various sites, each leading to an increased risk of cancer [11]. 5α-Reductase isoenzymes as targets for EDs are important enzymes not only due to metabolizing testosterone and its precursor to peripherally active androgens [12], but also in biosynthesis of 5α-saturated C21 neuroactive steroids as allopregnanolone [13]. So far little is known about EDs action on this enzyme in brain.

3β-Hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) are key enzymes involved in androgen biosynthesis in Leydig cells. Various phthalates were tested as potential inhibitors of these enzymes in human and rat testicular preparations. Their inhibitory activities differed according to the length of carbon chains in the ethanol moieties [14]. Both enzymes were also inhibited by perfluorinated chemicals [15]. For a review of the effect of a broad spectrum of EDs covering industrial materials (perfluoroalkyl compounds, phthalates, bisphenol A and benzophenone) and pesticides/biocides (methoxychlor, organotins, 1,2-dibromo-3-chloropropane and prochloraz) and plant constituents (genistein and gossypol) see e.g. [9].

17β-HSD and 11β-hydroxysteroid dehydrogenase (11β-HSD) are also enzymes regulating the actual concentration of biologically active hormonal steroids in peripheral tissues. While 17β-HSD acts on sex steroids (testosterone and estradiol and its 17-oxo precursors), 11β-HSD isoenzymes convert 11-oxo corticoids into their hormonally active 11β-hydroxy-derivatives and vice versa. While the first type of 11β-HSD acting as reductase is ubiquitous, the especial role of Type 2 11β-HSD acting as oxidase, protects kidney and also testicular Leydig cells from an excess of

Please cite this article in press as: R. Hampl, et al., Steroids and endocrine disruptors—History, recent state of art and open questions, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.04.013

glucocorticoid. Both hydroxysteroids dehydrogenases are targets for various endocrine disrupting chemicals, which may affect their activity as well as expression [16–18].

Another target for EDs are isoenzymes responsible for the sulfation of estrogen, androgens and their precursors in biosynthetic pathway. Sulfation plays an important role both in detoxification and in the control of steroid activity and, moreover, in the case of some so-called neurosteroids as e.g. dehydroepiandrosterone, it changes its activity the opposite way. Sulfotransferases were inhibited by phthalates, chlorinated phenols and also by some phytoestrogens [19–21].

An important step in the steroid hormone biosynthesis is the transport of cholesterol substrate across the inner mitochondrial membrane, mediated by Steroidogenic Active Regulatory protein (StAR) identified ten years ago [22]. StAR is present in mitochondria of typical steroidogenic cells as in adrenocortex and in gonads. Its gene belongs to those, the expression of which may also be inhibited by various EDs [23–25]. Besides "typical" EDs its expression at least in fish was also inhibited by heavy metal, namely cadmium [26].

The selection of the reviews and original papers should reflect the large publication activity in this field and is far from complete: for instance, as this chapter was being prepared there were as many as 150 references alone under the key words EDs and estrogen biosynthesis and review.

2.1. EDs and receptor-mediated steroid action

Steroid hormones exert their actions in the target cell through intracellular or membrane receptors. The classical genomic mechanism employing intracellular receptors consists of regulation – stimulation or inhibition – of the expression of particular genes. The individual steps started by hormone binding to the intracellular receptor, followed by the translocation of the steroid–receptor complex to the nuclei, its binding to steroid regulated elements and transactivation making accessible the initiation sites for mRNA transferase(s) and the initiation of transcription, actually includes a number of protein–protein interactions with nuclear factors (often called co-activators or co-repressors), many of which have only recently been revealed [27,28]. EDs may interfere with all these steps, leading to incredible diversity and complexity of EDs action. The investigation of EDs interaction with these transcription factors opens an interesting new field in the mosaic of their actions.

Regulatory mechanisms mediated by membrane receptors are not so frequent and, in most instances reported to date, deal with reproductive functions mediated by estrogen, progestine and androgen receptors, but examples concerning other steroid action have been reported as well; for the review see e.g. [29–31]. These mechanisms involve the binding of the steroid to a membrane receptor coupled with a G-protein, the activation of an effector (usually adenylate cyclase) and the initiation of a signaling cascade (usually phosphorylation), resulting in a variety of activation/inhibition effects.

Of interest are newly discovered "mixed mechanisms", beginning with steroid binding to a membrane receptor and initiating a signaling cascade, the final step of which is the activation of a so-called cAMP activated regulatory protein (CREB), which binds to the gene-regulatory elements and thus promotes transcription as in the "classical" mechanism; for examples see [32,33]. EDs may interfere with such a mechanism, too [34].

The methods of investigating how EDs interfere with receptor-mediated steroid action underwent remarkable progress) over the past few decades. For a review of these approaches see e.g. Adler in the cited monograph [35]. The first experiments in the early nineties dealt with the binding of EDs to steroid receptors as ligands and their competition with native steroid hormones. Various sources of steroid receptors from animal or human tissues or cell cultures were used. The great deal of reports concerned estrogen receptors (ERs), since most of the effects of EDs consisted of their estrogenic/antiestrogenic properties. The experimental data were compared with observed in vivo effects. It was clear that the binding of EDs to the respective steroid receptors differs in their affinity and kinetics, and that the binding characteristics need not correspond to the final disrupting effect.

Another way of investigating EDs actions consisted of studying the effects of EDs on gene expression. Steroid hormones regulate thousands of genes and a great advance brought the methods enabling the screening of a large array of expressed genes after the isolation and multiplication of cDNA from various hormone-responsive tissue preparations. The more detailed follow-up of gene expression in time and dose–response studies enabled methods employing gene reporters (e.g. luciferase) co-transfected with hormone response elements (HRE) of selected steroid-responsive gene. It was enabled by advances in molecular biology in the eighties, crowned by the characterization of human and animal genome. The remarkable differences in response to various steroid-receptor ligands led to a more detailed investigation of the events that followed steroid/disruptor binding to the respective receptors and the mapping of the signaling cascades and individual components involved. As mentioned above, these are very current topics and an interesting finding may be expected.

The differences in the effects of disruptors on various targets and more detailed knowledge of steroid receptor structures led to computer modeling of suitable ligand structures. This approach known as in silico methods (≡computer simulation) [36,37], in fact a virtual screening, is an inexpensive tool for studying quantitative structure activity relationships and docking, which undoubtedly belongs to perspective approaches in investigating EDs actions.

3. EDs and steroid hormone binding in circulation

Reversible binding to plasma transport proteins considerably influences the availability of free, biologically active, steroid hormones. Of particular importance are corticosteroid-binding globulin (CBG, transcortin) and sex hormone binding-globulin (SHBG). The structure and genes coding for these proteins have been well characterized. The main source of both proteins is the liver, but their expression is not limited to this tissue. Though fulfilling similar function, there is no homology between them. For the review covering the main information about their properties and functions see e.g. [38–41]. The SHBG function is broader: it possesses its own receptors on the membrane of cells from some reproductive tissues and triggers a signaling cascade by way of cAMP [41]. The binding of various non-steroidal ligands to SHBG could considerably modulate steroid hormone action; SHBG and CBG thus represent possible targets for various endocrine disruptors [42]. Surprisingly, the only reports on the interaction of EDs with the SHBG analog concerned fish [43], but analogous interaction of EDs was described for the thyroid binding protein transthyretin [44]. The reports on the effects of EDs on steroid-binding transport proteins have been scarce to date and new data may be expected.

3.1. How EDs may influence hypothalamic–pituitary hormonal axes on central levels

Steroid hormones, in concert with other hormones, genes, neurotransmitters, growth and other factors play an important role in the development and function of the brain and neural system in general. Since embryonic life, any of these well coordinated and programmed sequence of events may be affected or even

Please cite this article in press as: R. Hampl, et al., Steroids and endocrine disruptors—History, recent state of art and open questions, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.04.013

disrupted by environmental agents. Especially vulnerable are prenatal or early postnatal periods and puberty. Changes in brain development, including the central nervous system, caused by exposure to toxic and endocrine-disrupting agents may have serious consequences for the entire life, especially for reproductive functions, behavior, motor activity and cognition; for the review see e.g. [45]. In the following text we will focus only on the mechanisms by which EDs influence the central–peripheral regulations of steroid actions through major hypothalamic–pituitary–peripheral axes.

Gonadal and adrenal steroid hormone biosynthesis and secretion is controlled by feed-back mechanisms known as hypothalamic–pituitary–adrenal- or gonadal axes (HPA, HPG). Steroids interact with hypothalamic as well as pituitary receptors of the trophic cells. Both genomic and non-genomic mechanisms may take part there. This results in the secretion or biosynthesis of hypothalamic liberines or pituitary hormones. Various EDs can compete as antagonists or agonists with natural steroid hormones for the receptors thanks to their structural similarity or, in a genomic way by affecting their expression in various neuronal or pituitary cells.

The mechanisms are the same as those that occur in other target cells for steroid hormones. Some recent examples of ways how EDs intervene with hypothalamic–pituitary regulations are shown here. The cited papers demonstrate on animal models how EDs affect further development of hypothalamic–pituitary regulation system in prenatal or neonatal period(s): even low doses of bisphenol A administration in early life altered sex-specific ER expression in rat hypothalamus and amygdala [46]. Besides ER, bisphenol A also disrupted hypothalamic gonadoliberin (LHRH) mRNA processing in hypothalamic nuclei, both leading to changes in estrous cyclicity in adult rats [47]. Similarly acted PCBs, which altered the expression of a set of genes including estrogen receptors alpha in rat hypothalamus, also resulting in a change in the trajectory of postnatal development [48].

Bisphenol A also disrupted non-genomic estrogen induced signaling via ERs coupled with G-protein [49].

Among the protein actors expressed in the hypothalamus and pituitary, of particular importance is kisspeptin/GPR54 system. Kisspeptins, encoded by the *KISS1* gene, act as stimulators for hormone-induced gonadotropin secretion and their expression is regulated by sex steroids via a feed-back mechanism. Kisspeptin is now believed to be one of the key factors triggering puberty in mammals [50]. It is not surprising that it is a potential target for EDs and first reports brought evidence that various EDs affect its expression and function; for examples see [51,52]. Organochlorine EDs as DDT and its metabolites may even increase gonadoliberin secretion through its effects on steroid and glutamate receptors [53].

Compared with the HPG axis, less attention was paid to the effect of EDs on the HPA axis, for the review see e.g. [54]. Interestingly, the effect of atrazine, a widely used herbicide on LH release was in the first line mediated by stimulation of HPA axis and the alteration of adrenal hormone secretion. Among other things, it demonstrates a close linkage between both hypothalamic–pituitary–adrenal- and gonadal axes [55].

The examples shown here, performed mostly on animal models, demonstrate the diverse actions of EDs on hypothalamic and pituitary levels; further studies, especially in humans, are needed to complete the mosaic of EDs actions on the autonomous nervous system.

3.2. Steroids and endocrine disruptors in body fluids

Since EDs influence steroid biosynthesis and its actions at various levels, it logically raises the question of how steroid levels correlate with actual concentration of EDs. Advanced analytical techniques [56–58] enable the assessment of a large array of EDs and their metabolites (some of which may be more active than parental compounds) in various body fluids. The latter concerns not only blood serum (plasma) or urine, but also follicular fluid, semen, maternal milk, saliva and even tissues obtained by biopsy. The EDs content in male semen (seminal plasma, fluid) is of particular interest with respect to the observed decline of sperm quality within the last decades [59,60].

Two ways of investigation may be traced: how steroid levels in the body associate with concentration of EDs and how exposure to EDs influences actual steroid levels. Both studies of the general population and special cohorts of humans exposed to EDs risks were reported.

In Table 1 we attempted to summarize chronologically major reports on EDs from the last decade and, if measured too, steroids, in serum, urine and also maternal milk [61–63] and follicular fluid [64]. Besides general population from various geographic regions and including also pregnant women, the studies focused on various common disorders associated with hormonal imbalance as ovarian dysfunction and PCOS [65–67]. The most frequent analytes were bisphenol A and PCBs. A consensus was reached on the positive association of BPA levels with biomarkers of PCOS including insulinoresistance [67] and hyperandrogenemia [65]. Surprisingly, as may be seen, there are relatively few reports on the simultaneous determination of EDs and hormonal steroids in the same biological material, above all in serum. In females it concerned pregnant women [68,69] and the already mentioned PCOS [66,67]. In men, two large studies provided evidence of the association of decreased testosterone levels with PCB and perfluorooctanesulfonic acid (PFOS) concentration, respectively [57,70]. More studies are needed to address the issue regarding the association of other EDs concentrations, especially persistent and steroid levels in the same material.

From the point of view of male reproduction, more attention was devoted to determining various EDs in seminal plasma and its association with the impairment of semen quality. This issue has been reviewed in the previously cited chapter of Hauser et al. in Gore's monograph [60]. Only a few reports, however, have dealt with the simultaneous determination of EDs and steroids in seminal plasma, though there was a clear association between actual steroid- and EDs concentration in this fluid. We have reviewed this matter recently [71].

4. Conclusive remarks and further perspectives

Three years ago a special issue of Journal of Steroid Biochemistry was devoted to endocrine disruptors as such, but many themes more or less dealt with steroids. To avoid overlapping, we tried in this issue to focus more on steroids and, at the same time, to point to news of this very vital topic.

As concerns steroid metabolizing enzymes, a promising topic is the effects of EDs on brain 5α-reductase with respect to its key role in metabolizing of progesterone to saturated metabolites, known to act as modulators of $GABA_A$ receptors. More information is needed about EDs effects on steroid transport to mitochondria and their influence on StAR protein.

Hand in hand in learning of steroid-triggered signaling cascades the effects of EDs on individual steps may be expected. Interesting would be the impact of EDs on pluripotent glucocorticoid actions in the light of their immunomodulatory, anti-inflammatory and apoptotic properties. The virtual screening of large sets of genes regulated by steroids and their affection by EDs could be a promising approach.

Very little is known about the effects of EDs on steroid binding to plasma proteins.

Please cite this article in press as: R. Hampl, et al., Steroids and endocrine disruptors—History, recent state of art and open questions, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.04.013

Table 1
Endocrine disruptors and steroids in body fluids.

Author, year	Ref.	Subjects	Analyzed material	EDs	Steroid(s) and hormones	Main findings
Takeuchi et al., 2004	[65]	47 normal and women with various ovarian dysfunctions	Serum (both hormones and EDs)	BPA	Testosterone, androstene-dione	Strong relationship between serum BPA and androgen concentrations
Wang et al., 2006	[68]	50 pregnant women aged 25–34 years, 3rd trimester	Serum and placental tissue (both hormones and EDs)	17 dioxin congeners, 12 dioxin-like PCBs, and 6 indicator PCBs	4- and 2-hydroxylated E2 metabolites	The ratio of 4- to 2-hydroxylated estradiol decreased with increasing exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, while levels of 4-OH-estradiol increased with increasing concentrations of high-chlorinated dibenzofurans
Mocarelli et al., 2008	[72]	135 males (3 age groups) exposed in the past to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin	Serum (both hormones and EDs)	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Estradiol, FSH	Exposure in either period leads to permanent reduction of estradiol and increased FSH
Brucker-Davis et al., 2008	[61]	Nursing mothers of 6246 boys screened for cryptorchidism (1.6% cryptorchic)	Colostrum, 125 samples (56 for cryptorchid and 69 for controls)	15 antiandrogenic and/or antiestrogenic EDs including DDE, PCBs and others		All maternal milk available was contaminated with EDs, insignificantly higher concentrations were found in milk from mother of cryptorchid boys
Goncharov et al., 2009	[57]	Native Americans (Mohawks), 257 adult men and 436 women	Serum (both hormones and EDs)	PCBs – 101 congeners	Testosterone	Elevation in serum PCB levels is associated with a lower concentration of serum testosterone in men only
Bushnik et al., 2010	[73]	5319 general population	Serum, urine	BPA, lead		Blood lead was found in 100% samples, urinary BPA in 91%
Galloway et al., 2010	[74]	715 adults between 20 and 74 years of age	Urinary excretion and serum (steroids only)	BPA	Testosterone, estradiol, SHBG	No associations with the serum hormone levels and urinary BPA excretion, with exception of association between BPA and SHBG concentrations in premenopausal women
Weldon et al., 2010	[75]	366 low-income, Mexican-American pregnant women	Serum	PCB congeners, DDT, DDE		Persistent organic pollutants were not associated with shortened lactation duration, but may be associated with longer lactation duration
Wan et al., 2010	[69]	26 pregnant women and 28 matching fetuses	Serum (both hormones and EDs)	10 polybrominatd diphenyl ethers plus BPA	Estradiol	Concentrations of 6-hydroxylated polybrominated diphenyl ethers in maternal and cord serum were positively correlated, being significantly greater in cord blood serum
Kandaraki et al., 2011	[66]	71 women with PCOS and 100 healthy women	Serum (both hormones and EDs)	BPA	Testosterone, androstene-dione	Higher BPA levels in PCOS women than controls and a statistically significant positive association between androgens and BPA
Kadar et al., 2011	[62]	30 breast milk samples from French women	Breast milk	Perfluorinated compounds, PFOS, PFOA and other		Advanced analytics
Ye et al., 2012	[76]	936 children 3–11 years, general population	Serum, urine	BPA + 7 other phenols	Urine, not serum is the preferred matrix for EDs monitoring	
Gyllenhammar et al., 2012	[77]	100 young women, general population	Serum	Nonylphenol, BPA		Association with nutritional habits
Petro et al., 2012	[64]	40 women undergoing IVF	Folicullar fluid, serum	PCB 153 and other PCBs, p,p'DDE	Estradiol	Higher EDC contamination in the follicular fluid was associated with a decreased fertilization rate
Kim et al., 2012	[63]	Healthy women at delivery and their newborns, 21 pairs	Umbilical cord and maternal blood, breast milk	Polybrominated EDs		A strong correlation was found for studied EDs between breast milk and cord blood or maternal blood and cord blood samples
Meijer et al., 2012	[78]	53 pregnant women and their male newborns	Maternal serum at 35 weeks of pregnancy	8 neutral and 4 phenolic polychlorinated EDs		Organohalogen compounds correlated with markers of sexual development in boys up to 18 months of age
Joensen et al., 2012	[79]	881 healthy men, who provided serum, urine and semen samples	Serum (hormones), urine (EDs)	14 phthalate metabolites, including di(2-ethylhexyl)- and diisononyl-phthalate metabolites	Serum testosterone, estradiol, SHBG, LH, FSH, inhibin-B	Negative association of EDs with total and free testosterone and testosterone/estradiol ratio
Toft et al., 2012	[80]	588 partners of pregnant women who provided semen samples	Serum	4 perfluorinated chemicals		Negative associations between PFOS exposure and sperm morphology
Joensen et al., 2013	[70]	247 men, general population	Serum (both hormones and EDs)	Serfluorooctanesulfonate (PFOS)	Testosterone (total and free), LH, FSH	Negative association between serum PFOS and testosterone
Tarantino et al., 2013	[67]	40 women with PCOS and 20 healthy women	Serum (both hormones and EDs)	BPA	Testosterone, SHBG	Association of BPA with PCOS markers incl. insulinoresistance

Please cite this article in press as: R. Hampl, et al., Steroids and endocrine disruptors—History, recent state of art and open questions, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.04.013

Further studies are needed for a more complex understanding of the impact of EDs on major hypothalamic–pituitary–gonadal or adrenal axes and on the role of kisspeptin systems.

Finally, advanced analytical methods would enable the simultaneous assessment of EDs and a broad steroid spectrum in biological fluids and their association with various endocrine diseases. There is still a scarcity of data on the concentrations of EDs and steroids in the same body fluids. EDs and steroids could be measured not only in blood or urine, but also in seminal plasma, follicular fluid, cerebrospinal fluid and saliva, and new data may be expected.

Acknowledgment

The work was supported by the Grant no. 13369-4 from the Internal Grant Agency of the Czech Ministry of Health.

References

[1] A.K. Hotchkiss, C.V. Rider, C.R. Blystone, V.S. Wilson, P.C. Hartig, G.T. Ankley, P.M. Foster, C.L. Gray, L.E. Gray, Fifteen years after Wingspread—environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go, Toxicol. Sci. 105 (2) (2008) 235–259.
[2] A.C. Gore, in: A.C. Gore (Ed.), Endocrine-Disrupting Chemicals: From Basic Research to Clinical Practice, Humana Press, Totowa, NJ, 2010.
[3] M. Hill, J. Vrbikova, J. Zarubova, R. Kancheva, M. Velikova, L. Kancheva, J. Kubatova, M. Duskova, P. Marusic, A. Parizek, L. Starka, The steroid metabolome in lamotrigine-treated women with epilepsy, Steroids 76 (12) (2011) 1351–1357.
[4] J.T. Sanderson, The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals, Toxicol. Sci. 94 (1) (2006) 3–21.
[5] S.A. Whitehead, S. Rice, Endocrine-disrupting chemicals as modulators of sex steroid synthesis, Best Pract. Res. Clin. Endocrinol. Metab. 20 (1) (2006) 45–61.
[6] K. Svechnikov, G. Izzo, L. Landreh, J. Weisser, O. Soder, Endocrine disruptors and Leydig cell function, J. Biomed. Biotechnol. 2010 (2010) (pii: 684504).
[7] Z.R. Craig, W. Wang, J.A. Flaws, Endocrine-disrupting chemicals in ovarian function: effects on steroidogenesis, metabolism and nuclear receptor signaling, Reproduction 142 (5) (2011) 633–646.
[8] E.C. Bonefeld-Jorgensen, M. Long, M.V. Hofmeister, A.M. Vinggaard, Endocrine-disrupting potential of bisphenol A, bisphenol A dimethacrylate, 4-*n*-nonylphenol, and 4-*n*-octylphenol in vitro: new data and a brief review, Environ. Health Perspect. 115 (Suppl. 1) (2007) 69–76.
[9] L. Ye, Z.J. Su, R.S. Ge, Inhibitors of testosterone biosynthetic and metabolic activation enzymes, Molecules 16 (12) (2011) 9983–10001.
[10] K. Cheshenko, F. Pakdel, H. Segner, O. Kah, R.I. Eggen, Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish, Gen. Comp. Endocrinol. 155 (1) (2008) 31–62.
[11] B. Castro, P. Sanchez, J.M. Torres, O. Preda, R.G. del Moral, E. Ortega, Bisphenol A exposure during adulthood alters expression of aromatase and 5alpha-reductase isozymes in rat prostate, PLoS One 8 (2) (2013) e55905.
[12] S. Lo, I. King, A. Allera, D. Klingmuller, Effects of various pesticides on human 5alpha-reductase activity in prostate and LNCaP cells, Toxicol. In Vitro 21 (3) (2007) 502–508.
[13] A. Bali, A.S. Jaggi, Multifunctional aspects of allopregnanolone in stress and related disorders, Prog. Neuropsychopharmacol. Biol. Psychiatry 48 (2014) 64–78.
[14] K. Yuan, B. Zhao, X.W. Li, G.X. Hu, Y. Su, Y. Chu, B.T. Akingbemi, Q.Q. Lian, R.S. Ge, Effects of phthalates on 3beta-hydroxysteroid dehydrogenase and 17beta-hydroxysteroid dehydrogenase 3 activities in human and rat testes, Chem. Biol. Interact. 195 (3) (2012) 180–188.
[15] B. Zhao, Y. Chu, D.O. Hardy, X.K. Li, R.S. Ge, Inhibition of 3beta- and 17beta-hydroxysteroid dehydrogenase activities in rat Leydig cells by perfluorooctane acid, J. Steroid Biochem. Mol. Biol. 118 (1–2) (2010) 13–17.
[16] G.X. Hu, Q.Q. Lian, H. Lin, S.A. Latif, D.J. Morris, M.P. Hardy, R.S. Ge, Rapid mechanisms of glucocorticoid signaling in the Leydig cell, Steroids 73 (9–10) (2008) 1018–1024.
[17] M. Ohshima, S. Ohno, S. Nakajin, Inhibitory effects of some possible endocrine-disrupting chemicals on the isozymes of human 11beta-hydroxysteroid dehydrogenase and expression of their mRNA in gonads and adrenal glands, Environ. Sci. 12 (4) (2005) 219–230.
[18] J. Guo, X. Yuan, L. Qiu, W. Zhu, C. Wang, G. Hu, Y. Chu, L. Ye, Y. Xu, R.S. Ge, Inhibition of human and rat 11beta-hydroxysteroid dehydrogenases activities by bisphenol A, Toxicol. Lett. 215 (2) (2012) 126–130.
[19] R.H. Waring, S. Ayers, A.J. Gescher, H.R. Glatt, W. Meinl, P. Jarratt, C.J. Kirk, T. Pettitt, D. Rea, R.M. Harris, Phytoestrogens and xenoestrogens: the contribution of diet and environment to endocrine disruption, J. Steroid Biochem. Mol. Biol. 108 (3–5) (2008) 213–220.
[20] R. Harris, N. Turan, C. Kirk, D. Ramsden, R. Waring, Effects of endocrine disruptors on dehydroepiandrosterone sulfotransferase and enzymes involved in PAPS synthesis: genomic and nongenomic pathways, Environ. Health Perspect. 115 (Suppl. 1) (2007) 51–54.
[21] R.M. Harris, R.H. Waring, Sulfotransferase inhibition: potential impact of diet and environmental chemicals on steroid metabolism and drug detoxification, Curr. Drug Metab. 9 (4) (2008) 269–275.
[22] B.J. Clark, J. Wells, S.R. King, D.M. Stocco, The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR), J. Biol. Chem. 269 (45) (1994) 28314–28322.
[23] K.M. Caron, S.C. Soo, K.L. Parker, Targeted disruption of StAR provides novel insights into congenital adrenal hyperplasia, Endocr. Res. 24 (3–4) (1998) 827–834.
[24] B.J. Clark, R.K. Cochrum, The steroidogenic acute regulatory protein as a target of endocrine disruption in male reproduction, Drug Metab. Rev. 39 (2–3) (2007) 353–370.
[25] V. Kumar, C. Balomajumder, P. Roy, Disruption of LH-induced testosterone biosynthesis in testicular Leydig cells by triclosan: probable mechanism of action, Toxicology 250 (2–3) (2008) 124–131.
[26] N. Sandhu, M.M. Vijayan, Cadmium-mediated disruption of cortisol biosynthesis involves suppression of corticosteroidogenic genes in rainbow trout, Aquat. Toxicol. 103 (1–2) (2011) 92–100.
[27] H. Shibata, T.E. Spencer, S.A. Onate, G. Jenster, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action, Recent Prog. Horm. Res. 52 (1997) 141–164, discussion 164-145.
[28] D. Ratman, W. Vanden Berghe, L. Dejager, C. Libert, J. Tavernier, I.M. Beck, K. De Bosscher, How glucocorticoid receptors modulate the activity of other transcription factors: a scope beyond tethering, Mol. Cell. Endocrinol. 380 (1–2) (2013) 41–54.
[29] P. Thomas, Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models, Gen. Comp. Endocrinol. 175 (3) (2012) 367–383.
[30] S.L. Tilghman, E.N. Nierth-Simpson, R. Wallace, M.E. Burow, J.A. McLachlan, Environmental hormones: multiple pathways for response may lead to multiple disease outcomes, Steroids 75 (8–9) (2010) 520–523.
[31] S. De Coster, N. van Larebeke, Endocrine-disrupting chemicals: associated disorders and mechanisms of action, J. Environ. Public Health 2012 (2012) 713696.
[32] I. Quesada, E. Fuentes, M.C. Viso-Leon, B. Soria, C. Ripoll, A. Nadal, Low doses of the endocrine disruptor bisphenol-A and the native hormone 17beta-estradiol rapidly activate transcription factor CREB, FASEB J. 16 (12) (2002) 1671–1673.
[33] D. Grove-Strawser, M.I. Boulware, P.G. Mermelstein, Membrane estrogen receptors activate the metabotropic glutamate receptors mGluR5 and mGluR3 to bidirectionally regulate CREB phosphorylation in female rat striatal neurons, Neuroscience 170 (4) (2010) 1045–1055.
[34] C. Teng, B. Goodwin, K. Shockley, M. Xia, R. Huang, J. Norris, B.A. Merrick, A.M. Jetten, C.P. Austin, R.R. Tice, Bisphenol A affects androgen receptor function via multiple mechanisms, Chem. Biol. Interact. 203 (3) (2013) 556–564.
[35] S.R. Adler, Cellular mechanism of endocrine disruption, in: A.C. Gore (Ed.), Endocrine-Disrupting Chemicals: From Basic Research to Clinical Practice, Humana Press, Totowa, 2010, pp. 135–174.
[36] L.G. Nashev, A. Vuorinen, L. Praxmarer, B. Chantong, D. Cereghetti, R. Winiger, D. Schuster, A. Odermatt, Virtual screening as a strategy for the identification of xenobiotics disrupting corticosteroid action, PLoS One 7 (10) (2012) e46958.
[37] A. Vuorinen, A. Odermatt, D. Schuster, In silico methods in the discovery of endocrine disrupting chemicals, J. Steroid Biochem. Mol. Biol. 137 (2013) 18–26.
[38] L. Gagliardi, J.T. Ho, D.J. Torpy, Corticosteroid-binding globulin: the clinical significance of altered levels and heritable mutations, Mol. Cell. Endocrinol. 316 (1) (2010) 24–34.
[39] H.Y. Lin, Y.A. Muller, G.L. Hammond, Molecular and structural basis of steroid hormone binding and release from corticosteroid-binding globulin, Mol. Cell. Endocrinol. 316 (1) (2010) 3–12.
[40] G.V. Avvakumov, A. Cherkasov, Y.A. Muller, G.L. Hammond, Structural analyses of sex hormone-binding globulin reveal novel ligands and function, Mol. Cell. Endocrinol. 316 (1) (2010) 13–23.
[41] S.M. Kahn, D.J. Hryb, A.M. Nakhla, N.A. Romas, W. Rosner, Sex hormone-binding globulin is synthesized in target cells, J. Endocrinol. 175 (1) (2002) 113–120.
[42] D. Montes-Grajales, J. Olivero-Verbel, Computer-aided identification of novel protein targets of bisphenol A, Toxicol. Lett. 222 (3) (2013) 312–320.
[43] K.E. Tollefsen, Binding of alkylphenols and alkylated non-phenolics to the rainbow trout (*Oncorhynchus mykiss*) plasma sex steroid-binding protein, Ecotoxicol. Environ. Saf. 68 (1) (2007) 40–48.
[44] M. Montano, E. Cocco, C. Guignard, G. Marsh, L. Hoffmann, A. Bergman, A.C. Gutleb, A.J. Murk, New approaches to assess the transthyretin binding capacity of bioactivated thyroid hormone disruptors, Toxicol. Sci. 130 (1) (2012) 94–105.
[45] D.M. Walker, A.C. Gore, Endocrine-disrupting chemicals and the brain, in: A.C. Gore (Ed.), Endocrine-Disrupting Chemicals: From Basic Research to Clinical Practice, Humana Press, Totowa, NJ, 2010, pp. 63–109.
[46] J. Cao, M.E. Rebuli, J. Rogers, K.L. Todd, S.M. Leyrer, S.A. Ferguson, H.B. Patisaul, Prenatal bisphenol A exposure alters sex-specific estrogen receptor expression in the neonatal rat hypothalamus and amygdala, Toxicol. Sci. 133 (1) (2013) 157–173.
[47] L. Monje, J. Varayoud, M. Munoz-de-Toro, E.H. Luque, J.G. Ramos, Exposure of neonatal female rats to bisphenol A disrupts hypothalamic LHRH pre-mRNA processing and estrogen receptor alpha expression in nuclei controlling estrous cyclicity, Reprod. Toxicol. 30 (4) (2010) 625–634.

Please cite this article in press as: R. Hampl, et al., Steroids and endocrine disruptors—History, recent state of art and open questions, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.04.013

[48] S.M. Dickerson, S.L. Cunningham, A.C. Gore, Prenatal PCBs disrupt early neuroendocrine development of the rat hypothalamus, Toxicol. Appl. Pharmacol. 252 (1) (2011) 36–46.
[49] R. Vinas, C.S. Watson, Bisphenol S disrupts estradiol-induced nongenomic signaling in a rat pituitary cell line: effects on cell functions, Environ. Health Perspect. 121 (3) (2013) 352–358.
[50] L.F. Silveira, M.G. Teles, E.B. Trarbach, A.C. Latronico, Role of kisspeptin/GPR54 system in human reproductive axis, Front. Horm. Res. 39 (2010) 13–24.
[51] M. Bellingham, P.A. Fowler, M.R. Amezaga, S.M. Rhind, C. Cotinot, B. Mandon-Pepin, R.M. Sharpe, N.P. Evans, Exposure to a complex cocktail of environmental endocrine-disrupting compounds disturbs the kisspeptin/GPR54 system in ovine hypothalamus and pituitary gland, Environ. Health Perspect. 117 (10) (2009) 1556–1562.
[52] M. Tena-Sempere, Kisspeptin/GPR54 system as potential target for endocrine disruption of reproductive development and function, Int. J. Androl. 33 (2) (2010) 360–368.
[53] G. Rasier, A.S. Parent, A. Gerard, R. Denooz, M.C. Lebrethon, C. Charlier, J.P. Bourguignon, Mechanisms of interaction of endocrine-disrupting chemicals with glutamate-evoked secretion of gonadotropin-releasing hormone, Toxicol. Sci. 102 (1) (2008) 33–41.
[54] J.P. Hinson, P.W. Raven, Effects of endocrine-disrupting chemicals on adrenal function, Best Pract. Res. Clin. Endocrinol. Metab. 20 (1) (2006) 111–120.
[55] C.D. Foradori, L.R. Hinds, A.M. Quihuis, A.F. Lacagnina, C.B. Breckenridge, R.J. Handa, The differential effect of atrazine on luteinizing hormone release in adrenalectomized adult female Wistar rats, Biol. Reprod. 85 (4) (2011) 684–689.
[56] H. Gallart-Ayala, E. Moyano, M.T. Galceran, Recent advances in mass spectrometry analysis of phenolic endocrine disruptors and related compounds, Mass Spectrom. Rev. 29 (5) (2010) 776–805.
[57] A. Goncharov, R. Rej, S. Negoita, M. Schymura, A. Santiago-Rivera, G. Morse, D.O. Carpenter, Lower serum testosterone associated with elevated polychlorinated biphenyl concentrations in Native American men, Environ. Health Perspect. 117 (9) (2009) 1454–1460.
[58] M. Chen, L. Tao, E.M. Collins, C. Austin, C. Lu, Simultaneous determination of multiple phthalate metabolites and bisphenol-A in human urine by liquid chromatography–tandem mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 904 (2012) 73–80.
[59] E. Carlsen, A. Giwercman, N. Keiding, N.E. Skakkebaek, Evidence for decreasing quality of semen during past 50 years, Br. Med. J. 305 (6854) (1992) 609–613.
[60] R. Hauser, J.S. Barthold, J.D. Meeker, Epidemiologic evidence on the relationship between environmental endocrine disruptors and male reproductive and developmental health, in: A.C. Gore (Ed.), Endocrine Disrupting Chemicals: From Basic Research to Clinical Practice, Humana Press, Totowa, NJ, 2010, pp. 225–251.
[61] F. Brucker-Davis, B. Ducot, K. Wagner-Mahler, C. Tommasi, P. Ferrari, P. Pacini, M. Boda-Buccino, A. Bongain, P. Azuar, P. Fenichel, Environmental pollutants in maternal milk and cryptorchidism, Gynecol. Obstet. Fertil. 36 (9) (2008) 840–847.
[62] H. Kadar, B. Veyrand, A. Barbarossa, G. Pagliuca, A. Legrand, C. Bosher, C.Y. Boquien, S. Durand, F. Monteau, J.P. Antignac, B. Le Bizec, Development of an analytical strategy based on liquid chromatography–high resolution mass spectrometry for measuring perfluorinated compounds in human breast milk: application to the generation of preliminary data regarding perinatal exposure in France, Chemosphere 85 (3) (2011) 473–480.
[63] T.H. Kim, Y. Bang du, H.J. Lim, A.J. Won, M.Y. Ahn, N. Patra, K.K. Chung, S.J. Kwack, K.L. Park, S.Y. Han, W.S. Choi, J.Y. Han, B.M. Lee, J.E. Oh, J.H. Yoon, J. Lee, H.S. Kim, Comparisons of polybrominated diphenyl ethers levels in paired South Korean cord blood, maternal blood, and breast milk samples, Chemosphere 87 (1) (2012) 97–104.
[64] E.M. Petro, J.L. Leroy, A. Covaci, E. Fransen, D. De Neubourg, A.C. Dirtu, I. De Pauw, P.E. Bols, Endocrine-disrupting chemicals in human follicular fluid impair in vitro oocyte developmental competence, Hum. Reprod. 27 (4) (2012) 1025–1033.
[65] T. Takeuchi, O. Tsutsumi, Y. Ikezuki, Y. Takai, Y. Taketani, Positive relationship between androgen and the endocrine disruptor, bisphenol A, in normal women and women with ovarian dysfunction, Endocr. J. 51 (2) (2004) 165–169.
[66] E. Kandaraki, A. Chatzigeorgiou, S. Livadas, E. Palioura, F. Economou, M. Koutsilieris, S. Palimeri, D. Panidis, E. Diamanti-Kandarakis, Endocrine disruptors and polycystic ovary syndrome (PCOS): elevated serum levels of bisphenol A in women with PCOS, J. Clin. Endocrinol. Metab. 96 (3) (2011) E480–E484.
[67] G. Tarantino, R. Valentino, C. Di Somma, V. D'Esposito, F. Passaretti, G. Pizza, V. Brancato, F. Orio, P. Formisano, A. Colao, S. Savastano, Bisphenol A in polycystic ovary syndrome and its association with liver–spleen axis, Clin. Endocrinol. (Oxf.). 78 (3) (2013) 447–453.
[68] S.L. Wang, Y.C. Chang, H.R. Chao, C.M. Li, L.A. Li, L.Y. Lin, O. Papke, Body burdens of polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls and their relations to estrogen metabolism in pregnant women, Environ. Health Perspect. 114 (5) (2006) 740–745.
[69] Y. Wan, K. Choi, S. Kim, K. Ji, H. Chang, S. Wiseman, P.D. Jones, J.S. Khim, S. Park, J. Park, M.H. Lam, J.P. Giesy, Hydroxylated polybrominated diphenyl ethers and bisphenol A in pregnant women and their matching fetuses: placental transfer and potential risks, Environ. Sci. Technol. 44 (13) (2010) 5233–5239.
[70] U.N. Joensen, B. Veyrand, J.P. Antignac, M. Blomberg Jensen, J.H. Petersen, P. Marchand, N.E. Skakkebaek, A.M. Andersson, B. Le Bizec, N. Jorgensen, PFOS (perfluorooctanesulfonate) in serum is negatively associated with testosterone levels, but not with semen quality, in healthy men, Hum. Reprod. 28 (3) (2013) 599–608.
[71] R. Hampl, J. Kubátová, V. Sobotka, J. Heráček, Steroids in semen, their role in spermatogenesis and the possible impact of endocrine disruptors, Horm. Mol. Biol. Clin. Invest. 13 (2013) 1–5.
[72] P. Mocarelli, P.M. Gerthoux, D.G. Patterson Jr., S. Milani, G. Limonta, M. Bertona, S. Signorini, P. Tramacere, L. Colombo, C. Crespi, P. Brambilla, C. Sarto, V. Carreri, E.J. Sampson, W.E. Turner, L.L. Needham, Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality, Environ. Health Perspect. 116 (1) (2008) 70–77.
[73] T. Bushnik, D. Haines, P. Levallois, J. Levesque, J. Van Oostdam, C. Viau, Lead and bisphenol A concentrations in the Canadian population, Health Rep. 21 (3) (2010) 7–18.
[74] T. Galloway, R. Cipelli, J. Guralnik, L. Ferrucci, S. Bandinelli, A.M. Corsi, C. Money, P. McCormack, D. Melzer, Daily bisphenol A excretion and associations with sex hormone concentrations: results from the InCHIANTI adult population study, Environ. Health Perspect. 118 (11) (2010) 1603–1608.
[75] R.H. Weldon, M. Webster, K.G. Harley, A. Bradman, L. Fenster, M.D. Davis, A. Hubbard, D.B. Barr, N. Holland, B. Eskenazi, Serum persistent organic pollutants and duration of lactation among Mexican-American women, J. Environ. Public Health 2010 (2010) 861757.
[76] X. Ye, X. Zhou, L.Y. Wong, A.M. Calafat, Concentrations of bisphenol A and seven other phenols in pooled sera from 3–11 year old children: 2001–2002 National Health and Nutrition Examination Survey, Environ. Sci. Technol. 46 (22) (2012) 12664–12671.
[77] I. Gyllenhammar, A. Glynn, P.O. Darnerud, S. Lignell, R. van Delft, M. Aune, 4-Nonylphenol and bisphenol A in Swedish food and exposure in Swedish nursing women, Environ. Int. 43 (2012) 21–28.
[78] L. Meijer, A. Martijn, J. Melessen, A. Brouwer, J. Weiss, F.H. de Jong, P.J. Sauer, Influence of prenatal organohalogen levels on infant male sexual development: sex hormone levels, testes volume and penile length, Hum. Reprod. 27 (3) (2012) 867–872.
[79] U.N. Joensen, H. Frederiksen, M.B. Jensen, M.P. Lauritsen, I.A. Olesen, T.H. Lassen, A.M. Andersson, N. Jorgensen, Phthalate excretion pattern and testicular function: a study of 881 healthy Danish men, Environ. Health Perspect. 120 (10) (2012) 1397–1403.
[80] G. Toft, B.A. Jonsson, C.H. Lindh, A. Giwercman, M. Spano, D. Heederik, V. Lenters, R. Vermeulen, L. Rylander, H.S. Pedersen, J.K. Ludwicki, V. Zviezdai, J.P. Bonde, Exposure to perfluorinated compounds and human semen quality in Arctic and European populations, Hum. Reprod. 27 (8) (2012) 2532–2540.

Please cite this article in press as: R. Hampl, et al., Steroids and endocrine disruptors—History, recent state of art and open questions, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.04.013

PŘÍLOHA VI

Vitku J, Starka L, Bicikova M, Hill M, Heracek J, Sosvorova L, Hampl R: Endocrine disruptors and other inhibitors of 11beta-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition. J Steroid Biochem Mol Biol (2014) In press, available online. IF = 3.628

G Model
SBMB 4226 No. of Pages 10

ARTICLE IN PRESS

Journal of Steroid Biochemistry & Molecular Biology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition

Jana Vitku [a,*], Luboslav Starka [a], Marie Bicikova [a], Martin Hill [a], Jiri Heracek [b,c], Lucie Sosvorova [a], Richard Hampl [a]

[a] Institute of Endocrinology, Department of Steroids and Proteofactors, Prague, Czech Republic
[b] Charles University, Third Faculty of Medicine, Department of Urology, Prague, Czech Republic
[c] Faculty Hospital Kralovske Vinohrady, Department of Urology, Prague, Czech Republic

ARTICLE INFO

Article history:
Received 26 February 2014
Received in revised form 9 July 2014
Accepted 19 July 2014
Available online xxx

Keywords:
11β-hydroxysteroid dehydrogenase
Inhibitor
Endocrine disruptor
Testis
Colon
Adipose tissue
Brain
Placenta

ABSTRACT

Numerous chemicals in the environment have the ability to interact with the endocrine system. These compounds are called endocrine disruptors (EDs). Exposure to EDs represents one of the hypotheses for decreasing fertility, the increased risk of numerous cancers and obesity, metabolic syndrome and type 2 diabetes. There are various mechanisms of ED action, one of which is their interference in the action of 11β-hydroxysteroid dehydrogenase (11βHSD) that maintains a balance between active and inactive glucocorticoids on the intracellular level. This enzyme has two isoforms and is expressed in various tissues. Inhibition of 11βHSD in various tissues can have different consequences. In the case of EDs, the results of exposure are mainly adverse; on the other hand pharmaceutically developed inhibitors of 11βHSD type 1 are evaluated as an option for treating metabolic syndrome, as well as related diseases and depressive disorders. This review focuses on the effects of 11βHSD inhibitors in the testis, colon, adipose tissue, kidney, brain and placenta.

Contents

1. Introduction

Since anthropogenic substances have been introduced to the environment, people have been continuously exposed to those chemicals. It was assumed that they do not have any biological effects, yet many of them might in fact disrupt the endocrine system. Evidence indicates the impact of these chemicals – endocrine

* Correspondingauthor. Tel.: +420 224905238.
E-mail address: jkubatova@endo.cz (J. Vitku).

http://dx.doi.org/10.1016/j.jsbmb.2014.07.007
0960-0760/

Please cite this article in press as: J. Vitku, et al., Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.07.007

disruptors (EDs) – on all endocrine organs in a human or animal body [1].

The prevalence of obesity, type 2 diabetes, endocrine-related cancers (breast, endometrial, ovarian, prostate, testicular and thyroid cancers) increased worldwide in recent decades and also low semen quality has been the subject of discussion [1–3]. One of the hypotheses for decreasing fertility in some countries, increasing the risk of cancers, obesity and type 2 diabetes is exposure to EDs. The group of EDs currently consists of thousands of chemicals of differing structures, though plenty of other EDs are undoubtedly still waiting to be discovered.

EDs can act in numerous ways in an organism. The longest known ED mechanism of action is their binding to nuclear receptors. They can also modulate the enzyme system involved in e.g., steroidogenesis or the antioxidative system. An epigenetic mechanism was also reported [4–6].

Many EDs are known to interfere in the action of 11β-hydroxysteroid dehydrogenase isoenzymes (11βHSD1 and 11βHSD2), which maintain a balance between active and inactive glucocorticoids on the intracellular level. Many structurally distinct inhibitors of 11βHSD1 were developed in the pharmaceutical industry as potential therapeutic agents for treating obesity, metabolic syndrome, type 2 diabetes, Alzheimer's disease and major depressive disorder and associated diseases [7–10].

The isozymes of 11βHSD are distributed in numerous body tissues with various mRNA expressions and have different physiological effects. They have been shown to modulate glucocorticoid action mainly on autocrine and paracrine levels. With respect to the wide spectrum of local glucocorticoid actions, changes in intracellular glucocorticoid concentrations can have either favorable or deleterious effects.

This review focuses on EDs and other synthetically developed inhibitors of 11βHSD activity, and the consequences of their actions on the testis, brain, colon, adipose, kidney and placenta.

2. 11β-hydroxysteroid dehydrogenase (11βHSD)

11βHSD isoforms are important enzymes in steroid metabolism that regulate interconversion between active and inactive glucocorticoids. Conversion of cortisone to cortisol was first discovered more than 50 years ago [11]. The key role of this enzyme in the kidney was highlighted by Edwards et al. [12] and Funder et al. in their work [13]. They explained that mineralocorticoid receptors (MR) in distal nephrons are selective in vivo because of the presence of 11βHSD2, which inactivates the active stress hormone cortisol and allows aldosterone that is present in 100–1000 times lower concentrations than cortisol to bind to the MR. If 11βHSD2 activity is inhibited, cortisol will also bind to the receptor and a syndrome of apparent mineralocorticoid excess (AME) occurs.

Generally, two distinct isoforms of 11βHSD are distinguished: 11βHSD1 and 11βHSD2 (Table 1). 11βHSD1 is a bidirectional oxidoreductase but preferentially acts as a reductase in vivo. 11βHSD1 uses $NADP^+$/NADPH as a cofactor. The NADPH pool is available from hexose-6-phosphate dehydrogenase (H6PD), which catalyzes the formation of 6-phosphogluconate from glucose-6-phosphate in the pentose-phosphate pathway. Both enzymes are located in the lumen of endoplasmatic reticulum (ER) where functional as well as physical interaction has been demonstrated [14–16]. This co-localization results in cooperation [17], with high NADPH/NAD^+ ratio moving the 11βHSD1 reaction toward the reductase direction in most tissues [14].

On the other hand 11βHSD2 is unidirectional with NAD^+ as a cofactor. It is expressed not only in mineralocorticoid sensitive tissues such as kidney [18] and sweat glands [19] but also in the testis [20] or placenta [21]. Its role is to protect these tissues from an excess of glucocorticoids.

Both isozymes of 11βHSD are localized on plenty of sites in the body. 11βHSD1 is expressed in the liver, adipose, adrenal gland, brain, testis, ovary, vascular epithelium and eye [22–27]. 11βHSD2 is expressed in the kidney, colon, placenta, ovary, testis, salivary glands, sweat glands, pancreas, skin, lung, vascular endothelium [18,28–31].

In 1997, the third type of 11βHSD isozyme (11βHSD3) has been suggested in sheep kidneys [32]. Evolutionary analysis indicates that 11βHSD3 (also known as HSD11B1L, SCDR10B or SDR26C2) is the ancestor of 11βHSD1 [33]. It was reported that has orthologs in sea urchin, amphioxus and Ciona, thus, 11βHSD3 emerged before development of glucocorticoid signaling suggesting other physiological role for 11βHSD3 [34]. Moreover, 11βHSD3 exerts only weak dehydrogenase activity on the cortisol substrate with the presence of $NADP^+$ [35].

2.1. EDs and 11βHSD activities in testis

Both isoforms of 11βHSD are present in human testis with high levels of 11βHSD1 expression and lower levels of 11βHSD2 expression [24,30,36]. It seems that the expression of 11βHSD isozymes in testis differ across species; e.g., mice testis do not express neither 11βHSD1 nor 11βHSD2 [37]; weak expression of 11βHSD1 and almost zero expression of 11βHSD2 was found in chicken testis [38,39]. Moreover, there are ambiguities regarding the reaction direction of 11βHSD1 in Leydig cells [40–42] where also the switch from reductase activity to oxidase activity during maturation of rat Leydig cells has been reported [43]. This could be species-dependent as well because the switch has not been observed in pigs where dehydrogenase activity continuously predominates [44,45].

Some studies suggested enzymatic coupling between 11βHSD1 and 17β-hydroxysteroid dehydrogenase type 3 (17βHSD3) in testes [46,47]. However, a recent study has shown that the catalytic moiety of 17βHSD3 faces to cytoplasm and that is why it cannot share the ER-luminal NADP/NADPH pool with 11βHSD1 [48].

Stress conditions or inhibition of 11βHSD dehydrogenase activities results in a glucocorticoid excess in the Leydig cells. A surplus of glucocorticoids causes delayed genomic repression of testosterone production through GR or a rapid nongenomic decrease in testosterone production. The rapid depression has been hypothesized to occur via the putative plasma membrane corticosteroid receptor [46]. A decrease in testosterone levels leads afterwards to adverse changes on spermatogenesis. Therefore, it seems that 11βHSD 1 or 2 could be a target for EDs and this could be one of the possible mechanisms of endocrine disruption in the testis that leads to impaired spermatogenesis.

Many EDs have been detected in human body fluids such as blood plasma or urine [49]. Fewer chemicals were found in seminal fluid. Chemical substances that have been detected in human seminal fluid include phthalates: mono(2-ethylhexyl) phthalate (MEHP, di(2-ethylhexyl)phthalate (DEHP), polychlorinated biphenyls (PCBs), other organochlorine compounds (DDT and its metabolite p,p′-DDE organobromine compounds (polybrominated diphenyl esters), perfluorochemicals (perfluorooctanoic acid–PFOA), bisphenol A and dioxins (reviewed in [50]). Some of these compounds were found to

Table 1
Principal characteristics of two 11βHSD isozymes.

	11βHSD 1	11βHSD 2
Direction	Bidirectional	Unidirectional
Cofactor	$NADP^+$/NADPH	NAD^+
Distribution	Liver, adipose tissue	Kidney, testes
Affinity to substrate	Low	High

Please cite this article in press as: J. Vitku, et al., Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.07.007

Table 2
Inhibitors of 11βHSD1.

	Activity	Testing system	IC50 (μM)	Reference
Natural compounds				
Resveratrol	Reductase	Rat adipose microsomes	35.2	[51]
Curcumin	Reductase	CHOP cells microsomes (transfected with human HSD11B1)	10.62	[52]
		Rat testis microsomes	4.18	[52]
		Intact CHOP cells (transfected with human HSD11B1)	2.29	[52]
		Intact rat Leydig cells	5.78	[52]
(1E,4E)-1,5-Bis(3-methylthiophen-2-yl)penta-1,4-dien-3-one	Reductase	Intact rat Leydig cells	0.11	[52]
		Intact CHOP cells (transfected with human HSD11B1)	0.18	[52]
(1E,4E)-1,5-Bis(thiophen-2-yl)penta-1,4-dien-3-one	Reductase	Intact rat Leydig cells	0.18	[52]
		Intact CHOP cells (transfected with human HSD11B1)	0.09	[52]
(1E,4E)-1,5-Bis(thiophen-2-yl)cyclohexanone	Reductase	Intact rat Leydig cells	2.55	[52]
		Intact CHOP cells (transfected with human HSD11B1)	3.57	[52]
Emodin	Reductase	Intact 3T3-L1 adipocytes (1 h treatment)	7.24	[53]
		Intact 3T3-L1 adipocytes (24 h treatment)	4.2	[53]
		HEK 293 cells (transfected with human HSD11B1) microsomes	0.19	[54]
		HEK 293 cells (transfected with mouse HSD11B1) microsomes	0.09	[54]
Aloe emodin	Reductase	HEK 293 cells (transfected with human HSD11B1) microsomes	0.88	[54]
		HEK 293 cells (transfected with mouse HSD11B1) microsomes	0.1	[54]
Rheochrysidin	Reductase	HEK 293 cells (transfected with human HSD11B1) microsomes	0.54	[54]
		HEK 293 cells (transfected with mouse HSD11B1) microsomes	0.08	[54]
3-Methylchrysazin	Reductase	HEK 293 cells (transfected with human HSD11B1) microsomes	3.54	[54]
		HEK 293 cells (transfected with mouse HSD11B1) microsomes	0.4	[54]
Flavanone	Reductase	Lysates of HEK 293 cells (transfected with human HSD11B1)	18	[55]
		Intact HEK 293 cells (transfected with human HSD11B1)	8.3	[55]
		Intact 3T3-L1 adipocytes	13	[55]
6-Hydroxyflavanone	Reductase	Lysates of HEK 293 cells (transfected with human HSD11B1)	187	[55]
4′-Hydroxyflavanone	Reductase	Lysates of HEK 293 cells (transfected with human HSD11B1)	34	[55]
2′-Hydroxyflavanone	Reductase	Lysates of HEK 293 cells (transfected with human HSD11B1)	10	[55]
		Intact HEK 293 cells (transfected with human HSD11B1)	57	[55]
		Intact 3T3-L1 adipocytes	1.8	[55]
Abietic acid	Reductase	Lysates of HEK 293 cells (transfected with human HSD11B1)	27	[55]
	Oxidase	Lysates of HEK 293 cells (transfected with human HSD11B1)	2.8	[55]
Methyl jasmonate	Reductase	Lysates of HEK 293 cells (transfected with human HSD11B1)	107	[55]
Zearalenone	Oxidase	Lysates of HEK 293 cells (transfected with human HSD11B1)	84	[55]
Glycyrrhetinic acid	Reductase	Lysates of HEK 293 cells (transfected with human HSD11B1)	1.57	[55]
	Oxidase	Lysates of HEK 293 cells (transfected with human HSD11B1)	0.03	[55]
Euphane-3β,20-dihydroxy-24-ene	Reductase	HEK 293 cells (transfected with human HSD11B1) microsomes	0.04	[56]
		HEK 293 cells (transfected with mouse HSD11B1) microsomes	0.08	[56]
Kansuinone	Reductase	HEK 293 cells (transfected with human HSD11B1) microsomes	1.12	[56]
		HEK 293 cells (transfected with mouse HSD11B1) microsomes	1.08	[56]
Euphol	Reductase	HEK 293 cells (transfected with human HSD11B1) microsomes	0.02	[56]
		HEK 293 cells (transfected with mouse HSD11B1) microsomes	0.08	[56]
Kansenone	Reductase	HEK 293 cells (transfected with human HSD11B1) microsomes	0.003	[56]
		HEK 293 cells (transfected with mouse HSD11B1) microsomes	0.01	[56]
(24R)-Eupha-8,25-diene-3β,24-diol	Reductase	HEK 293 cells (transfected with human HSD11B1) microsomes	0.03	[56]
		HEK 293 cells (transfected with mouse HSD11B1) microsomes	0.05	[56]
(20R,23E)-Eupha-8,23-diene-3β,25-diol	Reductase	HEK 293 cells (transfected with human HSD11B1) microsomes	0.02	[56]
		HEK 293 cells (transfected with mouse HSD11B1) microsomes	0.3	[56]
Epigallocatechine-3-gallate	Oxidase	Human liver microsomes	131.2	[57]
	Reductase	Human liver microsomes	57.99	[57]
Environmental chemicals				
4-Nonylphenol	Reductase	Human liver microsomes	49.3	[36]
	Oxidase	Lysates of HEK 293 cells (transfected with human HSD11B1)	178	[55]
4-*t*-Octylphenol	Oxidase	Lysates of HEK 293 cells (transfected with human HSD11B1)	85	[55]
2,2′-Dihydroxybiphenyl	Oxidase	Lysates of HEK 293 cells (transfected with human HSD11B1)	151	[55]

Please cite this article in press as: J. Vitku, et al., Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.07.007

Table 2 (*Continued*)

	Activity	Testing system	IC50 (μM)	Reference
Dibenzoylmethane	Reductase	Lysates of HEK 293 cells (transfected with human HSD11B1)	52	[55]
Diethylhexyl adipate	Reductase	Human liver microsomes	49.4	[36]
BPA	Reductase	Human liver microsomes	14.81	[58]
		Rat testis microsomes	19.39	[58]
Methoxychlor	Reductase	Human liver microsomes	1.91	[59]
2-Bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane	Reductase	Human liver microsomes	8.88	[59]

inhibit 11βHSD activities (Tables 2 and 3), and probably a lot of EDs are still waiting to be discovered in seminal fluid.

Since some EDs were detected in seminal fluid and the mechanism of action through inhibition of 11βHSD dehydrogenase activities was also confirmed, this might lead to an excess of glucocorticoids in the Leydig cell, resulting in a testosterone decrease and eventually to spermatogenesis deterioration.

2.2. EDs and 11βHSD activities in colon

MRs are expressed in many tissues including the colon, and 11βHSD2 is also co-localized here to protect the MR from activation by glucocorticoids. 11βHSD2 mRNA levels are significantly increased in human colonic adenomas as well as $Apc^{+/min}$ mouse intestinal adenomas compared to levels in normal colonic tissues [65]. An abundance of 11βHSD2 activity leads to inactivation of a large amount of glucocorticoids, and inactive cortisone (or corticosterone in rodents) is therefore unable to activate GR. The lack of glucocorticoids subsequently releases the repression of inducible cyclooxygenase type 2 (COX-2), which is then expressed in high levels and promotes colon tumorogenesis [66].

Studies have shown that use of selective COX-2 inhibitors (such as rofecoxib) or nonselective COX inhibitors helps to reduce the number and size of colonic adenomas, although it is linked to increased cardiovascular risk [67,68] or increased gastrointestinal effects [69,70], respectively.

The inhibition of 11βHSD2 by glycyrrhizic acid (GA) suppresses COX-2 derived PGE2 production and colorectal tumor growth without adverse side effects of selective COX-2 inhibitors [65]. GA is an endocrine disruptor found in licorice roots and rhizomes. It is nonselective 11βHSD inhibitor and most of the GA is converted in the body into more potent metabolite, glycyrrhetinic acid (GE). The selective inhibition of 11βHSD2 is then a promising therapeutic option, particularly with the locally acting enteric 11βHSD2 inhibitor that is neither systemically absorbed nor influences renal 11βHSD2 [66]. Kratschmar et al. created 15 GA derivatives, that are highly selective and potent 11βHSD2 inhibitors, but their stability and tissue distribution still need to be examined [71].

Conversely, colorectal carcinoma (CRC) is associated with decreased levels of 11βHSD2 mRNA [72]. Similarly, 11βHSD2 activity in adenomas within the early stage of neoplastic transformation is downregulated. Therefore, downregulation of 11βHSD2 is a typical feature for the development of colorectal polypous lesions and their transformation into CRC [73]. At this stage of CRC development, GA treatment is likely to be ineffective.

2.3. EDs and 11βHSD activities in adipose tissue

Besides the liver and brain, 11βHSD1 is also expressed in adipose tissue [74] and regulates intracellular levels of glucocorticoids there. 11βHSD1 activity was found to be higher [75–77] or the same [78,79] in visceral adipose tissue than in subcutaneous adipose tissue. Moreover, a growing number of studies has shown an overexpression of adipose 11βHSD1 in obesity, type 2 diabetes and metabolic syndrome [76,78,80–82]. Similarly overexpression of H6PDH in adipose tissue of transgenic mice induce 11βHSD1 reductase activity leading to elevated local corticosterone production and mild accumulation of abdominal fat [83]. H6PDH then represents another possible target for intervention [84].

Interestingly, developmental difference has been detected between preadipocytes and adipocytes in 11βHSD1 directionality; 11βHSD1 in undifferentiated human omental adipose stromal cells exhibit primarily dehydrogenase activity opposed to mature omental adipocytes, where reductase activity predominates. The switch in 11βHSD1 activity occurs at early stage of differentiation process [77], probably as a result of increase in H6PDH mRNA levels [85].

EDs that inhibit 11βHSD1 dehydrogenase activity in preadipocytes are expected to increase local levels of active glucocorticoids, which are known to suppress proliferation in favor of differentiation preadipocytes to mature adipocytes [86,87]. That finding can be crucial in pathogenesis of obesity [77].

Multiple studies suggested that the inhibitor of 11βHSD1 is a potential therapeutic target for metabolic syndrome, obesity and type 2 diabetes treatment that acts by lowering intracellular levels of glucocorticoids [7,8,76,88–92]. For instance, the triterpenoids from *Euphorbia kansui* exhibit strong inhibitory activity against human 11βHSD1 that might be useful for therapeutic purposes to prevent type 2 diabetes [56].

One of the environmentally accessible compounds is resveratrol (Table 2). It is present e.g., in grapes and wine, berries and peanuts. It is a strong antioxidant and has a wide range of positive effects on an organism. Recently, significant inhibition of 11βHSD1 by resveratrol was observed in rat adipose microsomes, indicating one of the mechanisms of its antiobesity action [51]. Antiobese effects of resveratrol such as inhibiting preadipocyte differentiation, decreasing adipocyte proliferation, inducing adipocyte apoptosis, decreasing lipogenesis, and promoting lipolysis and β-oxidation of fatty acids was observed previously as well as recently [93–96]; improved steroidogenesis in Leydig cells in obese mice treated by resveratrol was also detected [97].

Studies with carbenoxolone, a synthetic 11βHSD inhibitor, showed increased insulin sensitivity and decreased glucose production in healthy men [98]. Furthermore, a reduced glucose production rate due to reduced glycogenolysis was observed. On the other hand, carbenoxolone as nonselective 11βHSD inhibitor increases intrarenal cortisol concentration by 11βHSD2 inhibition. Decreased activity of 11βHSD2 then leads to long-term side effects, including arterial hypertension [99]. The selective 11βHSD1 inhibitor should be clinically more useful. A promising 11βHSD1 inhibitor may also be able to better inhibit 11β-reductase activity than the opposite 11β-oxidase direction (for the review read [100]). There are also problems with the poor bioavailability in adipose tissue since inhibitors are highly distributed into the liver [101].

The well known endocrine disruptor bisphenol A (BPA) significantly inhibits human and rat 11βHSD1 activities in vitro with IC50s of 14.81 μM for human and 19.39 μM for rat, respectively [58] (see Table 2), but this mechanism of action in vivo due to pharmacological doses of the compound can be excluded. However, BPA can act through other mechanisms in

Please cite this article in press as: J. Vitku, et al., Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.07.007

Table 3
Inhibitors of 11βHSD2.

	Testing system	IC50 (μM)	Reference
Natural compounds			
Glycyrrhetinic acid	CHO cells (transfected with human HSD11B2) microsomes	0.01	[60]
	Lysates of HEK 293 cells (transfected with human HSD11B2)	0.03	[55]
	Lysates of HEK 293 cells (transfected with human HSD11B2)	0.4	[61]
	Intact HEK 293 cells (transfected with human HSD11B2)	1.01	[61]
Curcumin	Rat kidney microsomes	11.92	[52]
	Human kidney microsomes	14.56	[52]
(1E,4E)-1,5-Bis(3-methylthiophen-2-yl)penta-1,4-dien-3-one	Human kidney microsomes	19.58	[52]
Abietic acid	Lysates of HEK 293 cells (transfected with human HSD11B2)	12	[55]
Zearalenone	Lysates of HEK 293 cells (transfected with human HSD11B2)	107	[55]
Fusidic acid	Lysates of HEK 293 cells (transfected with human HSD11B2)	134	[55]
Euphane-3β,20-dihydroxy-24-ene	HEK 293 cells (transfected with human HSD11B2) microsomes	8.18	[56]
Kansuinone	HEK 293 cells (transfected with human HSD11B2) microsomes	2.63	[56]
Euphol	HEK 293 cells (transfected with human HSD11B2) microsomes	0.4	[56]
Kansenone	HEK 293 cells (transfected with human HSD11B2) microsomes	0.11	[56]
(24R)-Eupha-8,25-diene-3β,24-diol	HEK 293 cells (transfected with human HSD11B2) microsomes	1.69	[56]
(20R,23E)-Eupha-8,23-diene-3β,25-diol	HEK 293 cells (transfected with human HSD11B2) microsomes	0.67	[56]
Environmental and industry chemicals			
Carbenoxolone	CHO cells (transfected with human HSD11B2) microsomes	0.02	[60]
Endosulfan	Lysates of HEK 293 cells (transfected with human HSD11B2)	61	[55]
BPA	Lysates of HEK 293 cells (transfected with human HSD11B2)	50	[55]
Disulfiram	Lysates of HEK 293 cells (transfected with human HSD11B2)	0.13	[62]
Thiram	Lysates of HEK 293 cells (transfected with human HSD11B2)	0.13	[62]
Diethyldithiocarbamate (DEDTC)	Lysates of HEK 293 cells (transfected with human HSD11B2)	1.7	[62]
	Lysates of HEK 293 cells (transfected with human HSD11B2)	6.3	[55]
Pyrrolidine dithiocarbamate (PDTC)	Lysates of HEK 293 cells (transfected with human HSD11B2)	6.3	[62]
Maneb	Lysates of HEK 293 cells (transfected with human HSD11B2)	0.75	[62]
Zineb	Lysates of HEK 293 cells (transfected with human HSD11B2)	31	[62]
Diphenyltin	Lysates of HEK 293 cells (transfected with human HSD11B2)	1.42	[61]
	Intact HEK 293 cells (transfected with human HSD11B2)	2.89	[61]
Triphenyltin	Human kidney microsomes	3.3	[36]
	Lysates of HEK 293 cells (transfected with human HSD11B2)	3.19	[61]
	Intact HEK 293 cells (transfected with human HSD11B2)	0.99	[61]
Tributyltin	Human kidney microsomes	16.5	[36]
	Lysates of HEK 293 cells (transfected with human HSD11B2)	1.9	[61]
	Intact HEK 293 cells (transfected with human HSD11B2)	1.52	[61]
Dibutyltin	Lysates of HEK 293 cells (transfected with human HSD11B2)	1.95	[61]
	Intact HEK 293 cells (transfected with human HSD11B2)	5.03	[61]
4-*t*-Octylphenol	Human kidney microsomes	8.9	[36]
	Lysates of HEK 293 cells (transfected with HSD11B2)	30	[55]
4-Nonylphenol	Human kidney microsomes	20.3	[36]
	Lysates of HEK 293 cells (transfected with HSD11B2)	79	[55]
4-*n*-Octylphenol	Human kidney microsomes	23.5	[36]
4-*n*-Nonylphenol	Human kidney microsomes	26.2	[36]
Dicyclohexyl phtalate	Human kidney microsomes	46.5	[36]
	Rat kidney microsomes	32.64	[63]
Dipropyl phthalate	Rat kidney microsomes	85.59	[63]
Di-*n*-butyl phthlate	Rat kidney microsomes	13.69	[63]
Mono(2-ethylhexyl)phthalate	Rat kidney microsomes	121.8	[63]
Mono(2-ethylhexyl)phthalate	Human kidney microsomes	110.8	[63]
Perfluorooctyl sulphonate	Human kidney microsomes	0.05	[64]
	Rat kidney microsomes	0.29	[64]
Perfluorooctanoic acid	Human kidney microsomes	24.41	[64]
	Rat kidney microsomes	3.8	[64]
Perfluorohexanesulfonate	Human kidney microsomes	18.97	[64]
	Rat kidney microsomes	62.87	[64]
2-Bis(p-hydroxyphenyl)-1,1,1-trichloroethane	Human placental microsomes	55.57	[59]
	Rat kidney microsomes	12.96	[59]

Please cite this article in press as: J. Vitku, et al., Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.07.007

adipose tissue including an increase in the mRNA expression of 11βHSD1, peroxisome proliferator-activated receptor γ (PPARγ) and lipoprotein lipase (LPL) in omental adipose tissue samples and at visceral adipocytes. These results were observed even at the lowest concentration tested (10 nM) suggesting acceleration of adipogenesis [102].

There can be various mechanisms of action of each ED that can act in more than one way. This is the case of BPA that, besides alterations in 11βHSD activity and the activity of other enzymes, is known to interact with estrogen receptors (ER) [103], estrogen-related receptor γ [104,105], androgen [106,107], thyroid [108], glucocorticoid [109], PPARγ [110,111] and pregnane X receptor [112]. Signaling through non-classical ER was also reported [113]. Surprisingly, it is in this way that BPA induces human adipocyte differentiation rather than through GR activation [114].

With its effects, BPA belongs to the special group of endocrine disruptors that disrupt the balance of lipid metabolism and are called obesogens. These chemicals promote obesity by increasing the number of fat cells, up-regulating fat storage into existing fat cells, changing the amount of calories burned at rest, shifting energy balance to favor storage of calories or altering the mechanisms through which the body regulates appetite and satiety [115]. They have the potential to disrupt multiple metabolic signaling pathways, including dysregulation of 11βHSD1 [102,116]. However, the best documented mechanism of obesogen action is PPARγ activation, which is a key regulator of adipogenesis [115].

2.4. EDs and 11βHSD activities in kidney

Renal 11βHSD2 is highly expressed in distal tubules and collecting ducts as well as MRs [117]. Since MR is a non-selective receptor, it can bind not only mineralocorticoid aldosterone but also active glucocorticoids (cortisol in primates and corticosterone in rodents). 11βHSD2 inactivates active glucocorticoids to their inactive 11-oxo forms that cannot bind to the MR. This colocalization in vivo therefore allows aldosterone to bind to MR [13]. Aldosterone stimulates sodium resorption, and potassium excretion. A defective 11βHSD2 gene results in AME syndrome that is manifested by hypertension and hypokalemia. The same symptoms occur when 11βHSD2 is inhibited. Licorice has long been known to inhibit 11βHSD2 and induce AME like symptoms [118]. More 11βHSD2 inhibitors with the potential to have similar effects have been discovered to date (Table 3).

2.5. EDs and 11βHSD activities in brain

We can find both isoforms of 11βHSD in the brain, but in different regions and with different levels of expression. 11βHSD2 was found to be expressed in the human amygdala, caudate nucleus, cerebellum, corpus callosum, hippocampus, spinal cord, and thalamus in concentrations that range from 10 to 100-fold lower than those in the adrenal [119].

The role of 11βHSD2 in adult brain seems to be to centrally control blood pressure and sodium appetite, apparently by a mechanism protecting MR from glucocorticoids and leaving available space for aldosterone. Unlike its limited expression in the adult brain, this isozyme is highly expressed in the fetal brain, where it is crucial for physiological maturation [120].

11βHSD1 is expressed in humans in the hypothalamus, hippocampus, prefrontal cortex, and cerebellum [121,122] suggesting that 11βHSD1 could be an important regulator of the HPA axis [120]. A chronic increase in plasma corticosterone is associated with cognitive decline and hippocampal atrophy in a subgroup of aged rodents [123]. Furthermore, aged 11βHSD1 knockout mice learned as well as young mice and avoided cognitive decline seen in most aged wild-type mice. Intrahippocampal corticosterone was significantly reduced in aged 11βHSD1 knockout mice in comparison to the wild type [123]. Moreover, aged mice after treatment with a selective 11βHSD1 inhibitor (UE1961) that crosses the blood–brain-barrier for 10 days improved spatial memory performance [124]. In humans, four weeks of carbenoxolone administration (100 mg, three times a day) improved verbal fluency in healthy elderly men with no changes in plasma cortisol, suggesting that the 11βHSD1 modulates HPA axis [121]. On the other hand, the genetic background also plays an important role in HPA axiś regulation [125]. Thus, the selective inhibitor of 11βHSD1 targeted to the brain seems to be a potential therapeutic agent and deserves further investigation.

ABT-384 as a potent selective 11βHSD1 inhibitor was recently evaluated for inhibition of 11βHSD1 in the central nervous system (CNS) in healthy male volunteers. Partial CNS 11βHSD1 inhibition and fully hepatic inhibition of 11βHSD1 was observed in 1 mg of ABT-384 administration per day and full inhibition in doses greater than 2 mg daily [10]. These results indicate that this inhibitor could be beneficial in the treatment of diseases where there is the need to decrease intracellular cortisol levels in CNS such as Alzheimer's disease or major depressive disorder.

2.6. EDs and 11βHSD activities in placenta

The distribution of 11βHSD isozymes differs across placental tissue, therefore the enzymes provide divergent amounts of bioactive glucocorticoids in various cell types. 11βHSD1 expression in placenta was localized exclusively to intermediate trophoblast and vascular endothelium where only weak expression was found compared to chorion [126]. Its function in these tissues remains unclear. Sun et al. hypothesize that 11βHSD1 reductase activity in vascular endothelium as well as in other blood vessels can influence vascular tone [126,127].

In contrast, 11βHSD2 is strongly expressed in syncytiotrophoblast, the outler layer of a placenta that is the closest to maternal compartment [60,128]. It is widely accepted that 11βHSD2 in placenta creates a protective barrier to prevent a fetus against a maternal active glucocorticoids [60,126,129,130]. Also differences between cortisol levels in umbilical vein and umbilical artery showed that cortisol for the most part does not pass the placenta (changes in corticoid levels and levels of other steroids reviewed in [131]).

Studies on pregnant rats treated by carbenoxolone, the potent nonselective inhibitor of 11βHSD activities showed decreased birth weight of the offspring and elevated blood pressure as well as hyperglycemia later in life [129,132,133]. In humans, birth weight is also inversely associated with systolic blood pressure (e.g., [134–136]). Inhibition of placental 11βHSD2 in carbenoxolone treated rats also resulted in downregulation of renal 11βHSD2 and hepatic 11βHSD1 in adult offspring [129]. The arterial hypertension could be partly explained by the decreased 11βHSD2 activity in kidney [137]. Furthermore, elevated basal hypothalamus-pituitary-adrenal axis (HPA) activity and anxiety in aversive situations have been reported if enzymatic disturbances in foetoplacental barrier occurred [138].

In conclusion, studies on rats showed that placental 11βHSD2 is crucial for normal fetus development. In the case of prenatal stress or inhibition of the placental 11βHSD2, the fetus is abundantly exposed to maternal glucocorticoids resulting in the adverse consequences such as fetal growth restriction, increased cardiovascular risk and neuroendocrinology and anxiety disorders later in life.

3. Conclusion

EDs and therapeutically used inhibitors of 11βHSD modulate local glucocorticoid action. With respect to the very broad effects

Please cite this article in press as: J. Vitku, et al., Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.07.007

of glucocorticoids in different tissues they may possess adverse or beneficial effects.

The inhibition of 11βHSD2 by EDs or other inhibitors leads to an excessive quantity of glucocorticoids that makes mineralocorticoids impossible to bind to MR in mineralocorticoid tissue, causes AME [12] and accelerates atherogenesis [139]. In glucocorticoid target tissues as testis and placenta, it causes decreased testosterone levels in the testis [20] and fetal development disorders [140], respectively.

On the other hand, selective inhibitors of 11βHSD1 are the beneficial ones and can possess positive effects in various tissues such as adipose tissue, the brain and may be good therapeutic agents in the treatment of obesity, metabolic syndrome, Alzheimer's disease or major depressive disorder. It is important to develop inhibitors that are specific to 11βHSD1 and specifically inhibit their reductase activity with no effects on oxidase action.

Some of the 11βHSD1 inhibitors are available in diet, like curcumin in spice turmeric, catechins from green tea or resveratrol in grapes and wine, respectively (Table 2). Apart from 11βHSD1 inhibition, they are known to act by a multiple mechanism in anti-obese action (for review see [141]). Results from human studies are inconsistent, but they could have favorable effects in obesity prevention. Therefore, further clinical trials are needed to shed light on this issue.

EDs can have multiple effects on an organism and it is better to try to decrease its exposure, e.g., by replacing BPA-containing containers with glass. Nowadays, it is impossible to avoid EDs in our environment. Thus, there is the need to develop new sensitive methods to unmask EDs and figure out how to protect ourselves from them, especially during fetal development or other critical windows of susceptibility.

Acknowledgement

This work was supported by grant IGA NT/13369 and NT/12349 from the Czech Ministry of Health.

References

[1] E. Diamanti-Kandarakis, J.P. Bourguignon, L.C. Giudice, R. Hauser, G.S. Prins, A. M. Soto, R.T. Zoeller, A.C. Gore, Endocrine-disrupting chemicals: an endocrine society scientific statement, Endocr. Rev. 30 (4) (2009) 293–342.
[2] E. Carlsen, A. Giwercman, N. Keiding, N.E. Skakkebaek, Evidence for decreasing quality of semen during past 50 years, BMJ 305 (6854) (1992) 609–613.
[3] A.M. Andersson, N. Jorgensen, K.M. Main, J. Toppari, E. Rajpert-De Meyts, H. Leffers, A. Juul, T.K. Jensen, N.E. Skakkebaek, Adverse trends in male reproductive health: we may have reached a crucial 'tipping point', Int. J. Androl. 31 (2) (2008) 74–80.
[4] M.D. Anway, A.S. Cupp, M. Uzumcu, M.K. Skinner, Epigenetic transgenerational actions of endocrine disruptors and male fertility, Science. 308 (5727) (2005) 1466–1469.
[5] J.A. Rusiecki, A. Baccarelli, V. Bollati, L. Tarantini, L.E. Moore, E.C. Bonefeld-Jorgensen, Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit, Environ. Health Perspect. 116 (11) (2008) 1547–1552.
[6] D.C. Dolinoy, D. Huang, R.L. Jirtle, Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development, Proc. Natl. Acad. Sci. U. S. A. 104 (32) (2007) 13056–13061.
[7] L. Wang, J. Liu, A. Zhang, P. Cheng, X. Zhang, S. Lv, L. Wu, J. Yu, W. Di, J. Zha, X. Kong, H. Qi, Y. Zhong, G. Ding, BVT. 2733, a selective 11beta-hydroxysteroid dehydrogenase type 1 inhibitor, attenuates obesity and inflammation in diet-induced obese mice, PLoS One 7 (7) (2012) p. e40056.
[8] J.S. Park, S.D. Rhee, W.H. Jung, N.S. Kang, H.Y. Kim, S.K. Kang, J.H. Ahn, K.Y. Kim, Anti-diabetic and anti-adipogenic effects of a novel selective 11beta-hydroxysteroid dehydrogenase type 1 inhibitor in the diet-induced obese mice, Eur. J. Pharmacol. 691 (1–3) (2012) 19–27.
[9] A. Tiwari, INCB-13739, an 11beta-hydroxysteroid dehydrogenase type 1 inhibitor for the treatment of type 2 diabetes, IDrugs 13 (4) (2010) 266–275.
[10] D.A. Katz, W. Liu, C. Locke, P. Jacobson, D.M. Barnes, R. Basu, G. An, M.J. Rieser, D. Daszkowski, F. Groves, G. Heneghan, A. Shah, H. Gevorkyan, S.S. Jhee, L. Ereshefsky, G.J. Marek, Peripheral and central nervous system inhibition of 11beta-hydroxysteroid dehydrogenase type 1 in man by the novel inhibitor ABT-384, Transl. Psychiatry 3 (2013) p. e295.
[11] D. Amelung, H.J. Hubener, L. Roka, G. Meyerheim, Conversion of cortisone to compound F, J. Clin. Endocrinol. Metab. 13 (9) (1953) 1125–1126.
[12] C.R. Edwards, P.M. Stewart, D. Burt, L. Brett, M.A. McIntyre, W.S. Sutanto, E.R. de Kloet, C. Monder, Localisation of 11beta-hydroxysteroid dehydrogenase – tissue specific protector of the mineralocorticoid receptor, Lancet 2 (8618) (1988) 986–989.
[13] J.W. Funder, P.T. Pearce, R. Smith, A.I. Smith, Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated, Science. 242 (4878) (1988) 583–585.
[14] A.G. Atanasov, L.G. Nashev, R.A. Schweizer, C. Frick, A. Odermatt, Hexose-6-phosphate dehydrogenase determines the reaction direction of 11beta-hydroxysteroid dehydrogenase type 1 as an oxoreductase, FEBS Lett. 571 (1–3) (2004) 129–133.
[15] A.G. Atanasov, L.G. Nashev, L. Gelman, B. Legeza, R. Sack, R. Portmann, A. Odermatt, Direct protein–protein interaction of 11beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase in the endoplasmic reticulum lumen, Biochim. Biophys. Acta. 1783 (8) (2008) 1536–1543.
[16] I.J. Bujalska, N. Draper, Z. Michailidou, J.W. Tomlinson, P.C. White, K.E. Chapman, E.A. Walker, P.M. Stewart, Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11beta-hydroxysteroid dehydrogenase type 1, J. Mol. Endocrinol. 34 (3) (2005) 675–684.
[17] G. Banhegyi, A. Benedetti, R. Fulceri, S. Senesi, Cooperativity between 11beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase in the lumen of the endoplasmic reticulum, J. Biol. Chem. 279 (26) (2004) 27017–27021.
[18] A.K. Agarwal, T. Mune, C. Monder, P.C. White, NAD(+)-dependent isoform of 11beta-hydroxysteroid dehydrogenase. Cloning and characterization of cDNA from sheep kidney, J Biol Chem. 269 (42) (1994) 25959–25962.
[19] S. Kenouch, M. Lombes, F. Delahaye, E. Eugene, J.P. Bonvalet, N. Farman, Human skin as target for aldosterone: coexpression of mineralocorticoid receptors and 11 beta-hydroxysteroid dehydrogenase, J. Clin. Endocrinol. Metab. 79 (5) (1994) 1334–1341.
[20] R.S. Ge, Q. Dong, E.M. Niu, C.M. Sottas, D.O. Hardy, J.F. Catterall, S.A. Latif, D.J. Morris, M.P. Hardy, 11{Beta}-hydroxysteroid dehydrogenase 2 in rat Leydig cells: its role in blunting glucocorticoid action at physiological levels of substrate, Endocrinology 146 (6) (2005) 2657–2664.
[21] R.W. Brown, K.E. Chapman, C.R. Edwards, J.R. Seckl, Human placental 11beta-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform, Endocrinology 132 (6) (1993) 2614–2621.
[22] V. Lakshmi, R.R. Sakai, B.S. McEwen, C. Monder, Regional distribution of 11beta-hydroxysteroid dehydrogenase in rat brain, Endocrinology 128 (4) (1991) 1741–1748.
[23] M. Bicikova, M. Hill, R. Hampl, L. Starka, Inhibition of rat renal and testicular 11beta-hydroxysteroid dehydrogenase by some antihypertensive drugs, diuretics, and epitestosterone, Horm. Metab. Res. 29 (9) (1997) 465–468.
[24] A.K. Tannin, C. Agarwal, M.I. New, P.C. White, The human gene for 11beta-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization, J. Biol. Chem. 266 (25) (1991) 16,653–16658.
[25] B.R. Walker, J.L. Yau, L.P. Brett, J.R. Seckl, C. Monder, B.C. Williams, C.R. Edwards, 11Beta-hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids, Endocrinology 129 (6) (1991) 3305–3312.
[26] R. Benediktsson, J.L. Yau, S. Low, L.P. Brett, B.E. Cooke, C.R. Edwards, J.R. Seckl, 11Beta-hydroxysteroid dehydrogenase in the rat ovary: high expression in the oocyte, J. Endocrinol. 135 (1) (1992) 53–58.
[27] S. Rauz, C.M. Cheung, P.J. Wood, M. Coca-Prados, E.A. Walker, P.I. Murray, P.M. Stewart, Inhibition of 11beta-hydroxysteroid dehydrogenase type 1 lowers intraocular pressure in patients with ocular hypertension, QJM 96 (7) (2003) 481–490.
[28] Z. Kyossev, P.D. Walker, W.B. Reeves, Immunolocalization of NAD-dependent 11beta-hydroxysteroid dehydrogenase in human kidney and colon, Kidney Int. 49 (1) (1996) 271–281.
[29] G. Hirasawa, H. Sasano, K. Takahashi, K. Fukushima, T. Suzuki, N. Hiwatashi, T. Toyota, Z.S. Krozowski, H. Nagura, Colocalization of 11beta-hydroxysteroid dehydrogenase type II and mineralocorticoid receptor in human epithelia, J. Clin. Endocrinol. Metab. 82 (11) (1997) 3859–3863.
[30] A.L. Albiston, V.R. Obeyesekere, R.E. Smith, Z.S. Krozowski, Cloning and tissue distribution of the human 11beta-hydroxysteroid dehydrogenase type 2 enzyme, Mol. Cell. Endocrinol. 105 (2) (1994) R11–17.
[31] R.E. Smith, P.J. Little, J.A. Maguire, A.N. Stein-Oakley, Z.S. Krozowski, Vascular localization of the 11beta-hydroxysteroid dehydrogenase type II enzyme, Clin. Exp. Pharmacol. Physiol. 23 (6–7) (1996) 549–551.
[32] E.P. Gomez-Sanchez, V. Ganjam, Y.J. Chen, D.L. Cox, M.Y. Zhou, S. Thanigaraj, C. E. Gomez-Sanchez, The sheep kidney contains a novel unidirectional, high affinity NADP(+)-dependent 11beta-hydroxysteroid dehydrogenase (11beta-HSD-3), Steroids 62 (5) (1997) 444–450.
[33] M.E. Baker, Evolutionary analysis of 11beta-hydroxysteroid dehydrogenase-type 1, -type 2 -type 3 and 17beta-hydroxysteroid dehydrogenase-type 2 in fish, FEBS Lett. 574 (1–3) (2004) 167–170.
[34] M.E. Baker, Evolution of 11beta-hydroxysteroid dehydrogenase-type 1 and 11beta-hydroxysteroid dehydrogenase-type 3, FEBS Lett. 584 (11) (2010) 2279–2284.
[35] C. Huang, B. Wan, B. Gao, S. Hexige, L. Yu, Isolation and characterization of novel human short-chain dehydrogenase/reductase SCDR10B which is highly

Please cite this article in press as: J. Vitku, et al., Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.07.007

expressed in the brain and acts as hydroxysteroid dehydrogenase, Acta Biochim. Pol. 56 (2) (2009) 279–289.
[36] M. Ohshima, S. Ohno, S. Nakajin, Inhibitory effects of some possible endocrine-disrupting chemicals on the isozymes of human 11beta-hydroxysteroid dehydrogenase and expression of their mRNA in gonads and adrenal glands, Environ. Sci. 12 (4) (2005) 219–230.
[37] X.L. Moore, I. Hoong, T.J. Cole, Expression of the 11beta-hydroxysteroid dehydrogenase 2 gene in the mouse, Kidney Int. 57 (4) (2000) 1307–1312.
[38] P. Klusonova, M. Kucka, I. Miksik, J. Bryndova, J. Pacha, Chicken 11beta-hydroxysteroid dehydrogenase type 2: partial cloning and tissue distribution, Steroids 73 (3) (2008) 348–355.
[39] P. Klusonova, M. Kucka, P. Ergang, I. Miksik, J. Bryndova, J. Pacha, Cloning of chicken 11beta-hydroxysteroid dehydrogenase type 1 and its tissue distribution, J. Steroid Biochem. Mol. Biol. 111 (3–5) (2008) 217–224.
[40] C.M. Leckie, L.A. Welberg, J.R. Seckl, 11Beta-hydroxysteroid dehydrogenase is a predominant reductase in intact rat Leydig cells, J. Endocrinol. 159 (2) (1998) 233–238.
[41] H.B. Gao, R.S. Ge, V. Lakshmi, A. Marandici, M.P. Hardy, Hormonal regulation of oxidative and reductive activities of 11beta-hydroxysteroid dehydrogenase in rat Leydig cells, Endocrinology 138 (1) (1997) 156–161.
[42] R.S. Ge, M.P. Hardy, Initial predominance of the oxidative activity of type I 11beta-hydroxysteroid dehydrogenase in primary rat Leydig cells and transfected cell lines, J. Androl. 21 (2) (2000) 303–310.
[43] R.S. Ge, D.O. Hardy, J.F. Catterall, M.P. Hardy, Developmental changes in glucocorticoid receptor and 11beta-hydroxysteroid dehydrogenase oxidative and reductive activities in rat Leydig cells, Endocrinology 138 (12) (1997) 5089–5095.
[44] V. Sharp, L.M. Thurston, R.C. Fowkes, A.E. Michael, 11Beta-hydroxysteroid dehydrogenase enzymes in the testis and male reproductive tract of the boar (*Sus scrofa domestica*) indicate local roles for glucocorticoids in male reproductive physiology, Reproduction 134 (3) (2007) 473–482.
[45] V. Sharp, L.M. Thurston, R.C. Fowkes, A.E. Michael, Expression and activities of 11betaHSD enzymes in the testes and reproductive tracts of sexually immature male pigs, J. Steroid Biochem. Mol. Biol. 115 (3–5) (2009) 98–106.
[46] G.-X. Hu, Q.-Q. Lian, H. Lin, S.A. Latif, D.J. Morris, M.P. Hardy, R.-S. Ge, Rapid mechanisms of glucocorticoid signaling in the Leydig cell, Steroids 73 (9–10) (2008) 1018–1024.
[47] S.A. Latif, M. Shen, R.S. Ge, C.M. Sottas, M.P. Hardy, D.J. Morris, Role of 11beta-OH–C(19) and C(21) steroids in the coupling of 11beta-HSD1 and 17beta-HSD3 in regulation of testosterone biosynthesis in rat Leydig cells, Steroids 76 (7) (2011) 682–689.
[48] B. Legeza, Z. Balazs, L.G. Nashev, A. Odermatt, The microsomal enzyme 17beta-hydroxysteroid dehydrogenase 3 faces the cytoplasm and uses NADPH generated by glucose-6-phosphate dehydrogenase, Endocrinology 154 (1) (2013) 205–213.
[49] R. Hauser, J.S. Barthold, J.D. Meeker, Epidemiologic evidence on the relationship between environmental endocrine disruptors and male reproductive and developmental health, in: A.C. Gore (Ed.), Endocrine-Disrupting Chemicals: From Basic Research to Clinical Practice, Humana Press, Totowa, NJ, 2010.
[50] R. Hampl, J. Kubatova, J. Heracek, V. Sobotka, L. Starka, Hormones and endocrine disruptors in human seminal plasma, Endocr. Reg. 47 (3) (2013) 149–158.
[51] N. Tagawa, S. Kubota, I. Kato, Y. Kobayashi, Resveratrol inhibits 11beta-hydroxysteroid dehydrogenase type 1 activity in rat adipose microsomes, J. Endocrinol. 218 (3) (2013) 311–320.
[52] G.X. Hu, H. Lin, Q.Q. Lian, S.H. Zhou, J. Guo, H.Y. Zhou, Y. Chu, R.S. Ge, Curcumin as a potent and selective inhibitor of 11beta-hydroxysteroid dehydrogenase 1: improving lipid profiles in high-fat-diet-treated rats, PLos One 8 (3) (2013) p. e49976.
[53] Y.J. Wang, S.L. Huang, Y. Feng, M.M. Ning, Y. Leng, Emodin, an 11beta-hydroxysteroid dehydrogenase type 1 inhibitor, regulates adipocyte function in vitro and exerts anti-diabetic effect in ob/ob mice, Acta Pharmacol. Sin. 33 (9) (2012) 1195–1203.
[54] Y. Feng, S.L. Huang, W. Dou, S. Zhang, J.H. Chen, Y. Shen, J.H. Shen, Y. Leng, Emodin, a natural product, selectively inhibits 11beta-hydroxysteroid dehydrogenase type 1 and ameliorates metabolic disorder in diet-induced obese mice, Br. J. Pharmacol. 161 (1) (2010) 113–126.
[55] R.A. Schweizer, A.G. Atanasov, B.M. Frey, A. Odermatt, A rapid screening assay for inhibitors of 11beta-hydroxysteroid dehydrogenases (11beta-HSD): flavanone selectively inhibits 11beta-HSD1 reductase activity, Mol. Cell. Endocrinol. 212 (1–2) (2003) 41–49.
[56] L.Y. Guo, H.P. He, Y. Leng, Z. Yang, X.J. Hao, Inhibition of 11b-HSD1 by tetracyclic triterpenoids from Euphorbia kansui, Molecules 11 (2012) 826–11838.
[57] J. Hintzpeter, C. Stapelfeld, C. Loerz, H.J. Martin, E. Maser, Green tea and one of its constituents, epigallocatechine-3-gallate, are potent inhibitors of human 11beta-hydroxysteroid dehydrogenase type 1, PLos One 9 (1) (2014) p. e84468.
[58] J. Guo, X. Yuan, L. Qiu, W. Zhu, C. Wang, G. Hu, Y. Chu, L. Ye, Y. Xu, R.S. Ge, Inhibition of human and rat 11beta-hydroxysteroid dehydrogenases activities by bisphenol A, Toxicol. Lett. 215 (2) (2012) 126–130.
[59] J. Guo, H. Deng, H. Li, Q. Zhu, B. Zhao, B. Chen, Y. Chu, R.S. Ge, Effects of methoxychlor and its metabolite 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane on 11beta-hydroxysteroid dehydrogenase activities in vitro, Toxicol. Lett. 218 (1) (2013) 18–23.
[60] R.W. Brown, K.E. Chapman, Y. Kotelevtsev, J.L. Yau, R.S. Lindsay, L. Brett, C. Leckie, P. Murad, V. Lyons, J.J. Mullins, C.R. Edwards, J.R. Seckl, Cloning and production of antisera to human placental 11 beta-hydroxysteroid dehydrogenase type 2, Biochem. J. 313 (Pt 3) (1996) 1007–1017.
[61] A.G. Atanasov, L.G. Nashev, S. Tam, M.E. Baker, A. Odermatt, Organotins disrupt the 11beta-hydroxysteroid dehydrogenase type 2-dependent local inactivation of glucocorticoids, Environ. Health Perspect. 113 (11) (2005) 1600–1606.
[62] A.G. Atanasov, S. Tam, J.M. Rocken, M.E. Baker, A. Odermatt, Inhibition of 11beta-hydroxysteroid dehydrogenase type 2 by dithiocarbamates, Biochem. Biophy. Res. Commun. 308 (2) (2003) 257–262.
[63] B. Zhao, Y. Chu, Y. Huang, D.O. Hardy, S. Lin, R.S. Ge, Structure-dependent inhibition of human and rat 11beta-hydroxysteroid dehydrogenase 2 activities by phthalates, Chem. Biol. Interact. 183 (1) (2010) 79–84.
[64] B. Zhao, Q. Lian, Y. Chu, D.O. Hardy, X.K. Li, R.S. Ge, The inhibition of human and rat 11beta-hydroxysteroid dehydrogenase 2 by perfluoroalkylated substances, J. Steroid Biochem. Mol. Biol. 125 (1–2) (2011) 143–147.
[65] M.Z. Zhang, J. Xu, B. Yao, H. Yin, Q. Cai, M.J. Shrubsole, X. Chen, V. Kon, W. Zheng, A. Pozzi, R.C. Harris, Inhibition of 11beta-hydroxysteroid dehydrogenase type II selectively blocks the tumor COX-2 pathway and suppresses colon carcinogenesis in mice and humans, J. Clin. Invest. 119 (4) (2009) 876–885.
[66] P.M. Stewart, S.M. Prescott, Can licorice lick colon cancer? J. Clin. Invest. 119 (4) (2009) 760–763.
[67] R.S. Bresalier, R.S. Sandler, H. Quan, J.A. Bolognese, B. Oxenius, K. Horgan, C. Lines, R. Riddell, D. Morton, A. Lanas, M.A. Konstam, J.A. Baron, Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial, N. Engl. J. Med. 352 (11) (2005) 1092–1102.
[68] D. Mukherjee, S.E. Nissen, E.J. Topol, Risk of cardiovascular events associated with selective COX-2 inhibitors, JAMA 286 (8) (2001) 954–959.
[69] M. Fornai, L. Antonioli, R. Colucci, C. Pellegrini, G. Giustarini, L. Testai, A. Martelli, A. Matarangasi, G. Natale, V. Calderone, M. Tuccori, C. Scarpignato, C. Blandizzi, NSAID-induced enteropathy: are the currently available selective COX-2 inhibitors all the same? J. Pharmacol. Exp. Ther. 348 (1) (2014) 86–95.
[70] C. Scarpignato, R.H. Hunt, Nonsteroidal antiinflammatory drug-related injury to the gastrointestinal tract: clinical picture, pathogenesis, and prevention, Gastroenterol. Clin. North Am. 39 (3) (2010) 433–464.
[71] D.V. Kratschmar, A. Vuorinen, T. Da Cunha, G. Wolber, D. Classen-Houben, O. Doblhoff, D. Schuster, A. Odermatt, Characterization of activity and binding mode of glycyrrhetinic acid derivatives inhibiting 11beta-hydroxysteroid dehydrogenase type 2, J. Steroid Biochem. Mol. Biol. 125 (1–2) (2011) 129–142.
[72] S. Zbankova, J. Bryndova, M. Kment, J. Pacha, Expression of 11beta-hydroxysteroid dehydrogenase types 1 and 2 in colorectal cancer, Cancer Lett. 210 (1) (2004) 95–100.
[73] M. Moravec, J. Svec, P. Ergang, V. Mandys, L. Rehakova, Z. Zadorova, J. Hajer, M. Kment, J. Pacha, Expression of 11beta-hydroxysteroid dehydrogenase type 2 is deregulated in colon carcinoma, Histol. Histopathol. (2013) .
[74] I.J. Bujalska, S. Kumar, P.M. Stewart, Does central obesity reflect Cushing's disease of the omentum? Lancet 349 (9060) (1997) 1210–1213.
[75] J.H. Goedecke, D.J. Wake, N.S. Levitt, E.V. Lambert, M.R. Collins, N.M. Morton, R. Andrew, J.R. Seckl, B.R. Walker, Glucocorticoid metabolism within superficial subcutaneous rather than visceral adipose tissue is associated with features of the metabolic syndrome in South African women, Clin. Endocrinol. 65 (1) (2006) 81–87.
[76] L. Alberti, A. Girola, L. Gilardini, A. Conti, S. Cattaldo, G. Micheletto, C. Invitti, Type 2 diabetes and metabolic syndrome are associated with increased expression of 11beta-hydroxysteroid dehydrogenase 1 in obese subjects, Int. J. Obes. (Lond.) 31 (12) (2007) 1826–1831.
[77] I.J. Bujalska, E.A. Walker, M. Hewison, P.M. Stewart, A switch in dehydrogenase to reductase activity of 11beta-hydroxysteroid dehydrogenase type 1 upon differentiation of human omental adipose stromal cells, J. Clin. Endocrinol. Metab. 87 (3) (2002) 1205–1210.
[78] S.K. Paulsen, S.B. Pedersen, S. Fisker, B. Richelsen, 11Beta-HSD type 1 expression in human adipose tissue: impact of gender, obesity, and fat localization, Obesity (Silver Spring) 15 (8) (2007) 1954–1960.
[79] G. Torres, M. Ferrario, V. Mericq, Differences in expression, content, and activity of 11beta-HSD1 in adipose tissue between obese men and women, ISRN Endocrinol. 2012 (2012) 787201.
[80] R.S. Lindsay, D.J. Wake, S. Nair, J. Bunt, D.E. Livingstone, P.A. Permana, P.A. Tataranni, B.R. Walker, Subcutaneous adipose 11beta-hydroxysteroid dehydrogenase type 1 activity and messenger ribonucleic acid levels are associated with adiposity and insulinemia in Pima Indians and Caucasians, J. Clin. Endocrinol. Metab. 88 (6) (2003) 2738–2744.
[81] K. Kannisto, K.H. Pietilainen, E. Ehrenborg, A. Rissanen, J. Kaprio, A. Hamsten, H. Yki-Jarvinen, Overexpression of 11beta-hydroxysteroid dehydrogenase-1 in adipose tissue is associated with acquired obesity and features of insulin resistance: studies in young adult monozygotic twins, J. Clin. Endocrinol. Metab. 89 (9) (2004) 4414–4421.
[82] E. Rask, T. Olsson, S. Soderberg, R. Andrew, D.E. Livingstone, O. Johnson, B.R. Walker, Tissue-specific dysregulation of cortisol metabolism in human obesity, J. Clin. Endocrinol. Metab. 86 (3) (2001) 1418–1421.
[83] Y. Wang, L.H. Liu Du, Y. Nagaoka, W.K. Fan Lutfy, T.C. Friedman, M. Jiang, Y. Liu, Transgenic overexpression of hexose-6-phosphate dehydrogenase in adipose tissue causes local glucocorticoid amplification and lipolysis in male mice, Am. J. Physiol. Endocrinol. Metab. 306 (5) (2014) E543–E551.

Please cite this article in press as: J. Vitku, et al., Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.07.007

[84] K.N. Hewitt, E.A. Walker, P.M. Stewart, Minireview: hexose-6-phosphate dehydrogenase and redox control of 11{beta}-hydroxysteroid dehydrogenase type 1 activity, Endocrinology 146 (6) (2005) 2539–2543.
[85] I.J. Bujalska, L.L. Gathercole, J.W. Tomlinson, C. Darimont, J. Ermolieff, A.N. Fanjul, P.A. Rejto, P.M. Stewart, A novel selective 11beta-hydroxysteroid dehydrogenase type 1 inhibitor prevents human adipogenesis, J. Endocrinol. 197 (2) (2008) 297–307.
[86] H. Hauner, G. Entenmann, M. Wabitsch, D. Gaillard, G. Ailhaud, R. Negrel, E.F. Pfeiffer, Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium, J. Clin. Invest. 84 (5) (1989) 1663–1670.
[87] F. Gregoire, C. Genart, N. Hauser, C. Remacle, Glucocorticoids induce a drastic inhibition of proliferation and stimulate differentiation of adult rat fat cell precursors, Exp. Cell Res. 196 (2) (1991) 270–278.
[88] N.M. Morton, J.R. Seckl, 11Beta-hydroxysteroid dehydrogenase type 1 and obesity, Front. Horm. Res. 36 (2008) 146–164.
[89] J.S. Scott, F.W. Goldberg, A.V. Turnbull, Medicinal chemistry of inhibitors of 11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1), J. Med. Chem. 57 (11) (2014) 4466–4486.
[90] P. Anagnostis, N. Katsiki, F. Adamidou, V.G. Athyros, A. Karagiannis, M. Kita, D. P. Mikhailidis, 11Beta-hydroxysteroid dehydrogenase type 1 inhibitors: novel agents for the treatment of metabolic syndrome and obesity-related disorders? Metabolism 62 (1) (2013) 21–33.
[91] C.D. Pereira, I. Azevedo, R. Monteiro, M.J. Martins, 11Beta-hydroxysteroid dehydrogenase type 1: relevance of its modulation in the pathophysiology of obesity, the metabolic syndrome and type 2 diabetes mellitus, Diabetes Obes. Metab. 14 (10) (2012) 869–881.
[92] A. Odermatt, L.G. Nashev, The glucocorticoid-activating enzyme 11beta-hydroxysteroid dehydrogenase type 1 has broad substrate specificity: physiological and toxicological considerations, J. Steroid Biochem. Mol. Biol. 119 (1–2) (2010) 1–13.
[93] S.H. Baek, H.J. Chung, H.K. Lee, R. D'Souza, Y. Jeon, H.J. Kim, S.J. Kweon, S.T. Hong, Treatment of obesity with the resveratrol-enriched rice DJ-526, Sci. Rep. 4 (2014) 3879.
[94] J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu, J.S. Allard, G. Lopez-Lluch, K. Lewis, P.J. Pistell, S. Poosala, K.G. Becker, O. Boss, D. Gwinn, M. Wang, S. Ramaswamy, K.W. Fishbein, R.G. Spencer, E.G. Lakatta, D. Le Couteur, R.J. Shaw, P. Navas, P. Puigserver, D.K. Ingram, R. de Cabo, D.A. Sinclair, Resveratrol improves health and survival of mice on a high-calorie diet, Nature 444 (7117) (2006) 337–342.
[95] S. Costa Cdos, F. Rohden, T.O. Hammes, R. Margis, J.W. Bortolotto, A.V. Padoin, C.C. Mottin, R.M. Guaragna, Resveratrol upregulated SIRT1, FOXO1, and adiponectin and downregulated PPARgamma1-3 mRNA expression in human visceral adipocytes, Obes. Surg. 21 (3) (2011) 356–361.
[96] W.J. Pang, S.D.L. Sun Bai, Y.J. Yang, G.S. Yang, [Effects of resveratrol on pig primary preadipocytes proliferation, differentiation and transcription expression of Sirt1 gene], Sheng Wu Gong Cheng Xue Bao 22 (5) (2006) 850–855.
[97] H.J. Wang, Q. Wang, Z.M. Lv, C.L. Wang, C.P. Li, Y.L. Rong, Resveratrol appears to protect against oxidative stress and steroidogenesis collapse in mice fed high-calorie and high-cholesterol diet, Andrologia (2014) .
[98] B.R. Walker, A.A. Connacher, R.M. Lindsay, D.J. Webb, C.R. Edwards, Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation, J. Clin. Endocrinol. Metab. 80 (11) (1995) 3155–3159.
[99] R.C. Andrews, O. Rooyackers, B.R. Walker, Effects of the 11beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone on insulin sensitivity in men with type 2 diabetes, J. Clin. Endocrinol. Metab. 88 (1) (2003) 285–291.
[100] K. Chapman, M. Holmes, J. Seckl, 11Beta-hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action, Physiol. Rev. 93 (3) (2013) 1139–1206.
[101] X.Y. Ye, D. Yoon, S.Y. Chen, A. Nayeem, R. Golla, R. Seethala, M. Wang, T. Harper, B.G. Sleczka, A. Apedo, Y.X. Li, B. He, M. Kirby, D.A. Gordon, J.A. Robl, Synthesis and structure-activity relationship of 2-adamantylmethyl tetrazoles as potent and selective inhibitors of human 11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1), Bioorg. Med. Chem. Lett. 24 (2) (2014) 654–660.
[102] J. Wang, B. Sun, M. Hou, X. Pan, X. Li, The environmental obesogen bisphenol A promotes adipogenesis by increasing the amount of 11beta-hydroxysteroid dehydrogenase type 1 in the adipose tissue of children, Int. J. Obes. (Lond.) 37 (7) (2013) 999–1005.
[103] G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.A. Gustafsson, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, Endocrinology 139 (10) (1998) 4252–4263.
[104] V. Delfosse, M. Grimaldi, A. le Maire, W. Bourguet, P. Bourguet, Nuclear receptor profiling of bisphenol-A and its halogenated analogues, Vitam. Horm. 94 (2014) 229–251.
[105] H. Okada, T. Tokunaga, X. Liu, S. Takayanagi, A. Matsushima, Y. Shimohigashi, Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor-gamma, Environ. Health Perspect. 116 (1) (2008) 32–38.
[106] H.J. Lee, S. Chattopadhyay, E.Y. Gong, R.S. Ahn, K. Lee, Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor, Toxicol. Sci. 75 (1) (2003) 40–46.
[107] C. Teng, B. Goodwin, K. Shockley, M. Xia, R. Huang, J. Norris, B.A. Merrick, A.M. Jetten, C.P. Austin, R.R. Tice, Bisphenol A affects androgen receptor function via multiple mechanisms, Chem. Biol. Interact. 203 (3) (2013) 556–564.
[108] K. Moriyama, T. Tagami, T. Akamizu, T. Usui, M. Saijo, N. Kanamoto, Y. Hataya, A. Shimatsu, H. Kuzuya, K. Nakao, Thyroid hormone action is disrupted by bisphenol A as an antagonist, J. Clin. Endocrinol. Metab. 87 (11) (2002) 5185–5190.
[109] R.M. Sargis, D.N. Johnson, R.A. Choudhury, M.J. Brady, Environmental endocrine disruptors promote adipogenesis in the 3T3-L1 cell line through glucocorticoid receptor activation, Obesity (Silver Spring) 18 (7) (2010) 1283–1288.
[110] A. Pereira-Fernandes, H. Demaegdt, K. Vandermeiren, T.L. Hectors, P.G. Jorens, R. Blust, C. Vanparys, Evaluation of a screening system for obesogenic compounds: screening of endocrine disrupting compounds and evaluation of the PPAR dependency of the effect, PLoS One 8 (10) (2013) e77481.
[111] Y.F. Wang, H.R. Chao, C.H. Wu, C.H. Tseng, Y.T. Kuo, T.C. Tsou, A recombinant peroxisome proliferator response element-driven luciferase assay for evaluation of potential environmental obesogens, Biotechnol. Lett. 32 (12) (2010) 1789–1796.
[112] Y. Sui, N. Ai, S.H. Park, J. Rios-Pilier, J.T. Perkins, W.J. Welsh, C. Zhou, Bisphenol A and its analogues activate human pregnane X receptor, Environ. Health Perspect. 120 (3) (2012) 399–405.
[113] P. Alonso-Magdalena, O. Laribi, A.B. Ropero, E. Fuentes, C. Ripoll, B. Soria, A. Nadal, Low doses of bisphenol A and diethylstilbestrol impair Ca^{2+} signals in pancreatic alpha-cells through a nonclassical membrane estrogen receptor within intact islets of Langerhans, Environ. Health Perspect. 113 (8) (2005) 969–977.
[114] J.G. Boucher, A. Boudreau, E. Atlas, Bisphenol A induces differentiation of human preadipocytes in the absence of glucocorticoid and is inhibited by an estrogen-receptor antagonist, Nutr. Diabetes 02 (2014) .
[115] A. Janesick, B. Blumberg, Minireview: PPARgamma as the target of obesogens, J. Steroid Biochem. Mol. Biol. 127 (1–2) (2011) 4–8.
[116] A. Janesick, B. Blumberg, Obesogens, stem cells and the developmental programming of obesity, Int. J. Androl. 35 (3) (2012) 437–448.
[117] Z. Krozowski, A.L. Albiston, V.R. Obeyesekere, R.K. Andrews, R.E. Smith, The human 11beta-hydroxysteroid dehydrogenase type II enzyme: comparisons with other species and localization to the distal nephron, J. Steroid Biochem. Mol. Biol. 55 (5–6) (1995) 457–464.
[118] B.R. Walker, C.R. Edwards, Licorice-induced hypertension and syndromes of apparent mineralocorticoid excess, Endocrinol. Metab. Clin. North Am. 23 (2) (1994) 359–377.
[119] L. Yu, D.G. Romero, C.E. Gomez-Sanchez, E.P. Gomez-Sanchez, Steroidogenic enzyme gene expression in the human brain, Mol. Cell. Endocrinol. 190 (1–2) (2002) 9–17.
[120] C.S. Wyrwoll, M.C. Holmes, J.R. Seckl, 11Beta-hydroxysteroid dehydrogenases and the brain: from zero to hero, a decade of progress, Front. Neuroendocrino. 32 (3) (2011) 265–286.
[121] T.C. Sandeep, J.L. Yau, A.M. MacLullich, J. Noble, I.J. Deary, B.R. Walker, J.R. Seckl, 11Beta-hydroxysteroid dehydrogenase inhibition improves cognitive function in healthy elderly men and type 2 diabetics, Proc. Natl. Acad. Sci. U. S. A. 101 (17) (2004) 6734–6739.
[122] P.H. Bisschop, M.J. Dekker, W. Osterthun, J. Kwakkel, J.J. Anink, A. Boelen, U.A. Unmehopa, J.W. Koper, S.W. Lamberts, P.M. Stewart, D.F. Swaab, E. Fliers, Expression of 11beta-hydroxysteroid dehydrogenase type 1 in the human hypothalamus, J. Neuroendocrinol. 25 (5) (2013) 425–432.
[123] J.R. Seckl, B.R. Walker, 11Beta-hydroxysteroid dehydrogenase type 1 as a modulator of glucocorticoid action: from metabolism to memory, Trends Endocrinol. Metab. 15 (9) (2004) 418–424.
[124] K. Sooy, S.P. Webster, J. Noble, M. Binnie, B.R. Walker, J.R. Seckl, J.L. Yau, Partial deficiency or short-term inhibition of 11beta-hydroxysteroid dehydrogenase type 1 improves cognitive function in aging mice, J. Neurosci. 30 (2010) 867–13872.
[125] R.N. Carter, J.M. Paterson, U. Tworowska, D.J. Stenvers, J.J. Mullins, J.R. Seckl, M.C. Holmes, Hypothalamic-pituitary-adrenal axis abnormalities in response to deletion of 11beta-HSD1 is strain-dependent, J. Neuroendocrinol. 21 (11) (2009) 879–887.
[126] K. Sun, K. Yang, J.R. Challis, Differential expression of 11beta-hydroxysteroid dehydrogenase types 1 and 2 in human placenta and fetal membranes, J. Clin. Endocrinol. Metab. 82 (1) (1997) 300–305.
[127] B.R. Walker, R. Best, Clinical investigation of 11beta-hydroxysteroid dehydrogenase, Endocr. Res. 21 (1–2) (1995) 379–387.
[128] Z. Krozowski, J.A. MaGuire, A.N. Stein-Oakley, J. Dowling, R.E. Smith, R.K. Andrews, Immunohistochemical localization of the 11beta-hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta, J. Clin. Endocrinol. Metab. 80 (7) (1995) 2203–2209.
[129] H. Saegusa, Y. Nakagawa, Y.J. Liu, T. Ohzeki, Influence of placental 11beta-hydroxysteroid dehydrogenase (11beta-HSD) inhibition on glucose metabolism and 11beta-HSD regulation in adult offspring of rats, Metabolism 48 (12) (1999) 1584–1588.
[130] B.E. Murphy, S.J. Clark, I.R. Donald, M. Pinsky, D. Vedady, Conversion of maternal cortisol to cortisone during placental transfer to the human fetus, Am. J. Obstet. Gynecol. 118 (4) (1974) 538–541.
[131] A. Hill, R. Paskova, M. Kanceva, J. Velikova, L. Kubatova, K. Kancheva, M. Adamcova, Z. Mikesova, M. Zizka, H. Koucky, V. Sarapatkova, P. Kacer, M. Meloun, A. Parizek, Steroid profiling in pregnancy: a focus on the human fetus, J. Steroid Biochem. Mol. Bio. 139 (2014) 201–222.

Please cite this article in press as: J. Vitku, et al., Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.07.007

[132] R.S. Lindsay, R.M. Lindsay, C.R. Edwards, J.R. Seckl, Inhibition of 11-beta-hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the offspring, Hypertension 27 (6) (1996) 1200–1204.
[133] R.S. Lindsay, R.M. Lindsay, B.J. Waddell, J.R. Seckl, Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: studies with the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone, Diabetologia 39 (11) (1996) 1299–1305.
[134] D.J. Barker, C. Osmond, J. Golding, D. Kuh, M.E. Wadsworth, Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease, BMJ 298 (6673) (1989) 564–567.
[135] E. Roberts, P. Wood, Birth weight and adult health in historical perspective: evidence from a New Zealand cohort, 1907–1922, Soc. Sci. Med. 107 (2014) 154–161.
[136] N. Bergvall, A. Iliadou, T. Tuvemo, S. Cnattingius, Birth characteristics and risk of high systolic blood pressure in early adulthood: socioeconomic factors and familial effects, Epidemiology 16 (5) (2005) 635–640.
[137] J.I. Tang, C.J. Kenyon, J.R. Seckl, M.J. Nyirenda, Prenatal overexposure to glucocorticoids programs renal 11beta-hydroxysteroid dehydrogenase type 2 expression and salt-sensitive hypertension in the rat, J. Hypertens. 29 (2) (2011) 282–289.
[138] L.A. Welberg, J.R. Seckl, M.C. Holmes, Inhibition of 11beta-hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring, Eur. J. Neurosci. 12 (3) (2000) 1047–1054.
[139] G.A. Deuchar, D. McLean, P.W. Hadoke, D.G. Brownstein, D.J. Webb, J.J. Mullins, K. Chapman, J.R. Seckl, Y.V. Kotelevtsev, 11beta-hydroxysteroid dehydrogenase type 2 deficiency accelerates atherogenesis and causes proinflammatory changes in the endothelium in apoe-/- mice, Endocrinology 152 (1) (2011) 236–246.
[140] A. Harris, J. Seckl, Glucocorticoids, prenatal stress and the programming of disease, Horm. Behav. 59 (3) (2011) 279–289.
[141] S. Wang, N. Moustaid-Moussa, L. Chen, H. Mo, A. Shastri, R. Su, P. Bapat, I. Kwun, C.L. Shen, Novel insights of dietary polyphenols and obesity, J. Nutr. Biochem. 25 (1) (2014) 1–18.

Please cite this article in press as: J. Vitku, et al., Endocrine disruptors and other inhibitors of 11β-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition, J. Steroid Biochem. Mol. Biol. (2014), http://dx.doi.org/10.1016/j.jsbmb.2014.07.007

PŘÍLOHA VII

Sosvorova L, Vitku J, Chlupacova T, Mohapl M, Hampl R: Determination of seven selected neuro- and immunomodulatory steroids in human cerebrospinal fluid and plasma. Steroids, 98, 1 – 8 (2014). IF = 2.639

Steroids 98 (2015) 1–8

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Determination of seven selected neuro- and immunomodulatory steroids in human cerebrospinal fluid and plasma using LC-MS/MS

Lucie Sosvorova [a], Jana Vitku [a], Tereza Chlupacova [a], Milan Mohapl [b], Richard Hampl [a,*]

[a] Institute of Endocrinology, Department of Steroids and Proteofactors, Narodni 8, 11694 Prague, Czech Republic
[b] Military University Hospital Prague, Department of Neurosurgery, U Vojenske nemocnice 1200, 16902 Prague, Czech Republic

ARTICLE INFO

Article history:
Received 11 August 2014
Received in revised form 31 December 2014
Accepted 23 January 2015
Available online 9 February 2015

Keywords:
LC–MS/MS
Dehydroepiandrosterone metabolites
11β-Hydroxysteroid dehydrogenase
Cerebrospinal fluid

ABSTRACT

Dehydroepiandrosterone (DHEA) and its 7-oxo- and 7-hydroxy-metabolites occurring in the brain are considered neurosteroids. Metabolism of the latter is catalysed by 11β-hydroxysteroid dehydrogenase (11β-HSD) which also interconverts cortisol and cortisone. The concurrent metabolic reaction to DHEA 7-hydroxylation is the formation of 16α-hydroxy-DHEA. The LC–MS/MS method using triple stage quadrupole-mass spectrometer was developed for simultaneous quantification of free DHEA, 7α-hydroxy-DHEA, 7β-hydroxy-DHEA, 7-oxo-DHEA, 16α-hydroxy-DHEA, cortisol and cortisone in human plasma and cerebrospinal fluid (CSF). The method employs 500 μL of human plasma and 3000 μL of CSF extracted with diethyl ether and derivatized with 2-hydrazinopyridine. It has been validated in terms of sensitivity, precision and recovery. In plasma, the following values were obtained: limit of detection: 2–50 pg/mL; limit of quantification: 5–140 pg/mL; within-day precision 0.58–14.58%; between-day precision: 1.24–13.89% and recovery: 85–113.2%). For CSF, the values of limit of detection: 2–28 pg/mL; limit of quantification: 6–94 pg/mL; within-day precision; 0.63–5.48%; between-day precision: 0.88–14.59% and recovery: 85.1–109.4% were acquired. Medians and concentration ranges of detected steroids in plasma and CSF are given in subjects with excluded normal pressure hydrocephalus ($n = 37$; 65–80 years). The method enables simultaneous quantification of steroids important for the estimation of 11β-HSD activity in human plasma and CSF. It will be helpful in better understanding various degenerative diseases development and progression.

1. Introduction

Dehydroepiandrosterone (DHEA) is an important endogenous steroid with a broad range of biological effects. It is synthesized in the adrenal cortex, gonads, brain and gastrointestinal tract and is a precursor of androgens and estrogens. DHEA is, together with its sulfated derivative, the most abundant circulating steroid in young adult humans. The levels of DHEA decline considerably with age and correlate with degenerative changes [1,2]. Significant portion of circulating DHEA is further metabolized to its 7-oxygenated derivatives – 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA in various tissues, including the liver and brain [3–5].

7α-OH-DHEA was first isolated and identified in 1959 by Okada [6] and soon thereafter by Stárka [7]. For a long time, 7-hydroxylated metabolites were considered physiologically inoperative. At the turn of the millennium, however, a number of publications appeared reporting antiglucocorticoid, immunomodulatory, neuroprotective, antioxidant and antiapoptotic effects of 7-hydroxylated DHEA metabolites [1,8–13].

7-oxo-DHEA was isolated from body fluid as early as 1954 [14] and later was established as a natural constituent of human plasma and urine [1]. In the 1990s it was reported that endogenous 7-oxo-metabolite of DHEA could act as an ergosteroid, enhancing the activity of several enzymes that influence a thermogenic system in rat liver [15,16]. Later also the neuroprotective and antiglucocorticoid actions of 7-oxo-DHEA were discovered [1,17,18].

The abovementioned 7-oxygenated steroids are metabolized by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1): 7α-OH-DHEA is a substrate for the 11β-HSD1, which

Abbreviations: DHEA, dehydroepiandrosterone; 7α-OH-DHEA, 7α-hydroxy-dehydroepiandrosterone; 7β-OH-DHEA, 7β-hydroxy-dehydroepiandrosterone; 16α-OH-DHEA, 16α-hydroxy-dehydroepiandrosterone; LC–MS/MS, liquid chromatography–tandem mass spectrometry; 11β-HSD, 11β-hydroxysteroid dehydrogenase; CSF, cerebrospinal fluid; UPLC, ultra-high pressure liquid chromatography; MRM, multiple reaction monitoring.

* Corresponding author.
E-mail address: rhampl@endo.cz (R. Hampl).

http://dx.doi.org/10.1016/j.steroids.2015.01.019
0039-128X/

converts 7α-OH-DHEA into 7β-OH-DHEA via 7-oxo-DHEA and also vice versa [19–21]. The 11β-HSD1 is mainly localized in glucocorticoid responsive tissues such as liver, brain, adipose tissue and others, where the NADP(H)-dependent oxido-reduction of cortisol to cortisone occurs, [19,21,22]. The modelling analyses indicated that 7-oxo and 7-OH-DHEA occupy the same binding site in 11β-HSD1 as cortisol and cortisone, respectively [23].

A concurrent metabolic reaction to 7-hydroxylation of DHEA is hydroxylation at carbon 16, resulting in 16-hydroxylated steroids [1]. 16α-OH-DHEA was first detected in humans in the 1950s [24], and only several reports concerning this steroid appeared later [25–29]. 16α-Hydroxylation of DHEA occurs in liver, adrenal cortex and also in several other tissues [30]. Increased levels of 16α-OH-DHEA were recently associated with autoimmune diseases and may counteract the beneficial effects of 7-oxo- and 7-hydroxylated-DHEA metabolites. The ratio of 16α-OH-, 7α-OH-, 7β-OH- and 7-oxo-DHEA in blood and various tissues may differ in healthy subjects and patients with autoimmune diseases and brain function disorders [29]. Recently, 16α-OH-DHEA was also detected in the cerebrospinal fluid (CSF) [31]. It indirectly supports the previous hypothesis of the role of 16α-OH-DHEA in brain function disorders.

This work presents the development and validation of a sensitive method, which involves liquid–liquid extraction, derivatization with 2-hydrazinopyridine and LC–MS/MS, for simultaneous quantification of DHEA, 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA, 16α-OH-DHEA, cortisol and cortisone in human plasma and CSF.

2. Experimental

2.1. Chemicals and reagents

Cortisol, cortisone and dehydroepiandrosterone (DHEA) were purchased form Koch-Light Laboratories LTD (Colnbrook, Great Britain), 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA, 16α-OH-DHEA and D3-DHEA were from Steraloids (Newport, USA). D4-Cortisol was from CDN isotopes (Ponte-Claire, Canada). 2-hydrazinopyridine, ammonium formate, methyl tert-butyl ether and trifluoroacetic acid were from Sigma–Aldrich (St. Louis, USA). LC–MS grade methanol, water and diethyl ether were from Merck AG (Darmstadt, Germany). The physiological solution (0.9% sodium chloride) was from B-Braun (Melsungen AG, Germany). [1,2,6,7–3H]Cortisol, specific radioactivity 3.04 TBq/mmol was from Amersham Biosciences, Inc. (Amersham, UK).

2.2. Preparation of stock solutions, calibration standards, and quality control samples

Stock solutions of steroids and deuterated steroids were prepared gravimetrically in methanol at the concentration of 1 mg/mL. Working standard solutions were prepared at 10 ng/mL and 1 ng/mL by diluting the stock solutions with methanol. The LC/MS standard solution was prepared by mixing appropriate volumes of individual steroid working solution and methanol to obtain a mixture containing 400 ng/mL cortisol, 80 ng/mL cortisone, 20 ng/mL DHEA, 3 ng/mL 7α-OH-DHEA and 7β-OH-DHEA, 1 ng/mL 7-oxo-DHEA and 16α-OH-DHEA for plasma samples. For the CSF samples, the LC/MS standard solution contained 100 ng/mL cortisol, 25 ng/mL cortisone, 2 ng/mL 7α-OH-DHEA, 1 ng/mL DHEA, 7β-OH-DHEA, 7-oxo-DHEA and 16α-OH-DHEA. All stock standards and LC/MS standards were stored at −20 °C and allowed to equilibrate at room temperature for at least 15 min before use. An eight-points calibration curve was prepared for calibration.

The stock internal standard solutions contained D3-DHEA and D4-cortisol (plasma: 10 ng/mL and 100 ng/mL, respectively; CSF: both 10 ng/mL). All standard and internal standard solutions were first pipetted into an empty tube and evaporated in a vacuum evaporator to dryness.

The calibration curve samples, zero samples (only internal standards added) and blank samples (no standards added) were prepared in duplicate similarly for the plasma and CSF samples, only by substituting plasma with charcoal-treated plasma and CSF with physiological solution. Calibration ranges for individual steroids are shown in Table 1. Quality control samples were prepared in-house, using a pool plasma and CSF with appropriate volumes of steroid stock solutions. Charcoal treated plasma was prepared by an in-house method employing multistep adsorption of steroids on charcoal. Absence of steroids was checked by spiking of plasma with [^{3}H]cortisol (10,000 dpm/mL) and measurement of remaining radioactivity close to zero.

2.3. Samples

Samples were previously collected from 37 subjects (64–85 years; 24 females and 13 males) tested for suspected normal pressure hydrocephalus, in which, however, this diagnosis was excluded on the basis of a combination of NMR imaging and lumbar drainage test [32]. Peripheral blood and CSF were collected in the morning before lumbar drainage test. The surgeries were

Table 1
The calibration ranges, correlation coefficients, limits of detection (LOD) and limits of quantification (LOQ), within-day and between-day coefficients of variability (*CV*) for individual steroids in plasma and cerebrospinal fluid.

Analyte	Calibration range (ng/mL)	Correlation coefficient (R^2)	LOD (pg/mL)	LOQ (pg/mL)	Within-day *CV* (%)	Between-day *CV* (%)
Plasma matrix						
DHEA	0.156–20	0.9995	5	15	0.58–6.31	3.46–4.24
Cortisol	3.125–400	0.9998	50	140	0.55–3.92	4.19–9.31
Cortisone	0.625–80	0.999	4	13	1.67–6.00	1.32–8.07
7α-OH-DHEA	0.023–3	0.9991	3	10	3.78–9.48	5.8–10.1
7β-OH-DHEA	0.023–3	0.9992	2	5	4.79–13.05	5.20–10.46
7-oxo-DHEA	0.008–1	0.9988	3	8	2.26–9.52	5.02–8.41
16α-OH-DHEA	0.008–1	0.9989	2	5	10.09–14.85	1.24–13.89
Cerebrospinal fluid matrix						
DHEA	0.008–1	0.9996	4	12	3.31–4.75	0.88–4.47
Cortisol	0.8–100	0.9994	28	94	0.81–2.38	3.09–5.05
Cortisone	0.2–25	0.999	9	30	0.85–4.44	4.74–9.5
7α-OH-DHEA	0.016–2	0.9993	12	40	2.32–4.32	1.85–4.50
7β-OH-DHEA	0.008–1	0.9994	2	6	2.09–2.42	2.75–6.48
7-oxo-DHEA	0.008–1	0.9989	12	40	4.28–5.48	4.45–14.59
16α-OH-DHEA	0.008–1	0.9989	5	16	0.63–3.42	1.82–7.99

performed in Department of Neurosurgery of Central Military Hospital in Prague.

Samples for the preparation of pooled plasma, charcoal-treated plasma and pooled CSF were the excessive amounts obtained from subjects investigated for the scientific purposed in the Institute of Endocrinology and Department of Neurosurgery of Central Military Hospital in Prague.

Samples were collected in plastic tubes, frozen and stored at −79 °C. The protocol was approved by the Ethical Committee of the Institute of Endocrinology. Informed and written consent with the use of biological materials for research reasons was obtained from all subjects participating to the project.

2.4. Optimization of extraction procedure, derivatization reaction and LC–MS/MS analysis

Methyl tert-butyl ether and diethyl ether in various volumes and extraction times were tested for the extraction. The diethyl ether extraction showed higher signals and better shapes of individual peaks. Various reaction volumes (50, 100 and 200 μL) and reaction times (10, 15, 20, 30, 45 and 60 min) were tested to obtain monoderivates with 2-hydrazinopyridine giving the highest intensities of the signal.

For the liquid chromatography, various mobile phases with and without formic acid (0.1%), mobile phase gradients, flow rates and column oven temperatures were used. For selected conditions, the best intensities and shapes of individual peaks were reached.

Optimal conditions were used for sample preparation and LC–MS/MS analysis.

2.5. Sample preparation

Plasma sample (500 μL) was spiked with 10 μL of stock internal standard solution and diluted with 500 μL of the physiological solution. Samples were extracted with 3 mL of diethyl ether to obtain free (unconjugated) steroids; the water phase was frozen in solid carbon dioxide and the organic phase was transferred into a glass tube and the solvent was evaporated. Control samples of the known concentration of individual analytes were processed in the same way.

CSF sample (3000 μL) was spiked with 20 μL of stock internal standard solution, diluted with 1000 μL of physiological solution and then extracted with 4 mL of diethyl ether, the water phase was frozen in solid carbon dioxide and the organic phase was transferred into a glass tube. The organic phase was evaporated. Control samples of the known concentration of individual analytes were processed in the same way.

In the next step 100 μL of derivatization solution was added. This solution was prepared freshly and contained 2-hydrazinopyridine, methanol and trifluoroacetic acid (1 mg: 5 mL: 1.63 μL) according to Higashi et al. [33]. The samples were mixed and incubated for 15 min at 60 °C. After incubation, the solvent was evaporated under stream of nitrogen.

The dry residues were dissolved in 100 μL of 10 mM ammonium formate solution in 50% methanol and mixed vigorously to rinse the tube walls. The samples were than centrifuged (2000*g*, 4 min, 22 °C). The whole amount of the solution was transferred to the insert vials and 50 μL, corresponding to 250 μL of plasma and 1500 μL of CSF, respectively, were injected into the UPLC.

2.6. LC–MS/MS conditions

LC–MS/MS was performed using an API 3200 (AB Sciex, Concord, Canada) triple stage quadrupole-mass spectrometer with electrospray ionization (ESI) connected to the UPLC Eksigent ultra-LC 110 system (Redwood City, CA, USA). Chromatographic separation was carried out on Kinetex C18 2.6 μm (150 × 3.0 mm) column (Phenomenex, Torrance, CA, USA) with a corresponding security guard at the flow rate 0.55 mL/min at 40 °C for plasma samples and at the flow rate 0.75 mL/min at 50 °C for CSF samples.

Mobile phases consisted of water (solvent A) and methanol (solvent B). The following gradient was employed (all steps linear): 0 min, 50:50 (A:B); 2 min, 50:50; 6 min, 10:90; 7 min, 5:95; 8 min 50 s, 5:95; 9 min, 50:50; 11 min, 50:50 and at 11 min stop for plasma samples and 0 min, 50:50 (A:B); 2 min, 50:50; 4 min, 25:75; 7 min, 5:95; 8.5 min 5:95; 9 min, 50:50; 10 min, 50:50 and at 10 min stop for CSF samples. LC mobile phase was diverted to waste till the 3rd minute and from the 9th minute of the run.

The mass spectrometer was operating in the positive ESI mode using multiple-reaction monitoring transitions (MRMs). The conditions were as follows: curtain gas: 25 psi, ion spray voltage: 5.5 kV, vaporizer temperature: 600 °C, ion source gas 1: 40 psi, ion source gas 2: 60 psi, interface heater: on. Nitrogen was produced by a high purity nitrogen generator (Peak Scientific instruments Ltd., model NM20Z, Renfrewshire, Scotland) and employed as curtain, nebulizer and collision gasses.

2.7. Quantification of the samples

The analytes were quantified by means of calibration curves made on the basis of known concentrations in the mixtures of analysed standards with constant level of internal standards. The calibration curves were obtained by plotting the response factor (-analyte area/internal standard area) against the concentration of the calibration standard. The values were corrected for procedural losses according to yields of internal standards. For DHEA, 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA and 16α-OH-DHEA D3-DHEA was used as internal standard. Cortisol and cortisone were quantified using the D4-cortisol as internal standard.

2.8. Method performance characteristics

Calibration curves were obtained by linear regression analysis using internal standardization. The data were fit to a linear least square regression curve with a weighing index of $1/x$. Calibration ranges and correlation coefficients for individual steroids are shown in Table 1. Peak area ratio between target analyte and its internal standard was used for quantitation. The assay acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value.

The limit of detection (LOD) and limit of quantification (LOQ) were defined according to Taverniers [34] as the concentrations at which the signal-to-noise ratio (S/N) was 3 and 10, respectively. They were determined by repetitive analyses ($n = 6$) of the lowest quality control samples independent of the calibration curve, which were stepwise diluted so that the S/N was around 10 and 3. The analyte response was compared to the zero sample response.

For the precision study, the concentrations of each analyte was determined six times during the same day (within-day precision) and six times on different days (between-day precision). The precision was expressed as relative standard deviation (RSD).

For the plasma samples, the recovery of the analytes was determined by spiking pooled plasma ($n = 5$) with three concentrations according to the physiological value of individual analytes. The method employing CSF, the recovery of the analytes was determined by spiking pooled CSF samples with two concentrations

Table 2
Mass spectrometric settings: precursor, quantification and confirmation ions, declustering potentials (DP), entrance potentials (EP), collision entrance potentials (CEP), collision energies (CE) and collision cell exit potentials (CXP) for measured analytes and deuterated internal standards and retention times (RT) of detected steroids in plasma and CSF.

Analyte	Precursor ion	Quantification ion	Confirmation ion	DP (V)	EP (V)	CEP (V)	CE (V)[a]	CXP (V)[a]	Plasma RT (min)	CSF RT (min)
DHEA	380	159	131	66	5	20	43 (53)	4	7.42	6.14
Cortisol	454	120	121	81	8.5	20	73 (53)	4	6.53	5.16
Cortisone	452	121	120	71	6.5	22	51 (51)	4	6.33	5.02
7α-OH-DHEA	369	134	115	66	11	20	45 (129)	4	6.42	5.07
7β-OH-DHEA	396	148	131	76	8	22	41 (45)	4	6.03	4.78
7-oxo-DHEA	394	131	107	71	8	18	51 (59)	4	6.11	4.86
16α-OH-DHEA	396	378	128	56	7.5	20	29 (29)	32 (4)	6.58	5.31
D4-Cortisol	458	120	121	91	7	22	71 (55)	4	6.54	5.19
D3-DHEA	383	162	107	66	9.5	16	43 (47)	4	7.43	6.12

[a] Values for the confirmation ion are given in the parentheses.

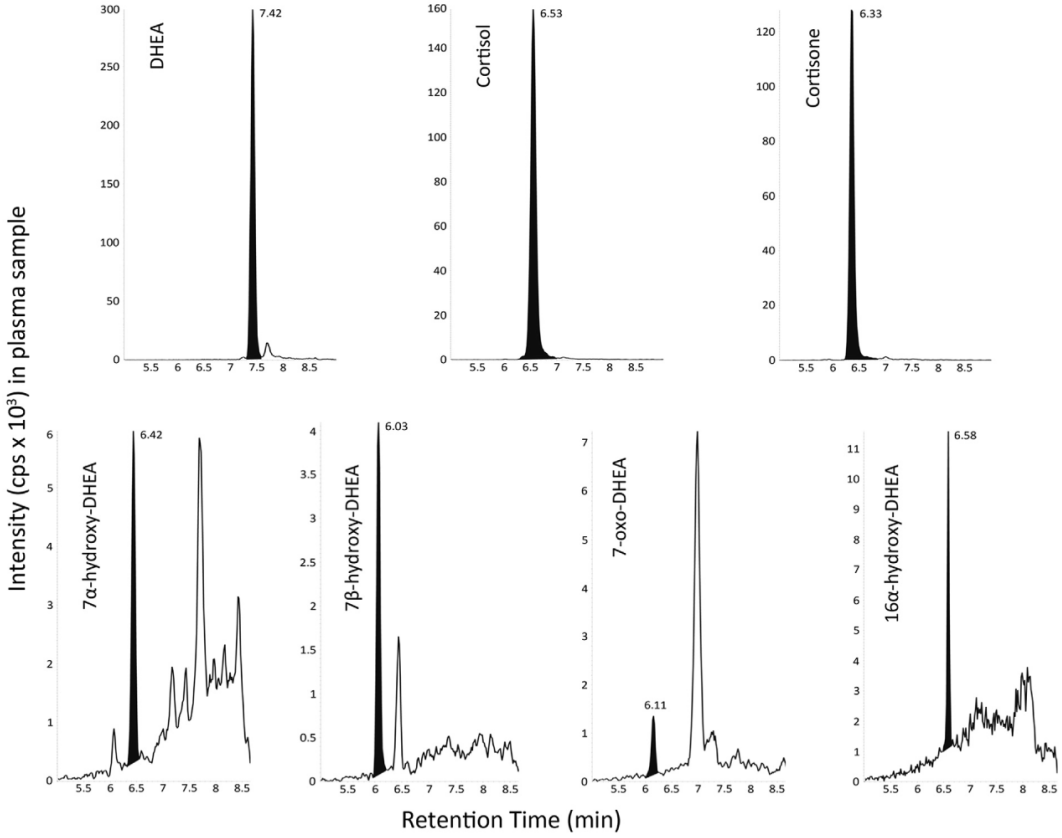


Fig. 1. Chromatograms of individual steroids in plasma sample. Transition details are presented in Table 2 and retention times (min) are indicated above the peak.

according to the physiological values of individual analytes. The spiking by only two concentrations was proceeded because of the limited amount of CSF matrix. The spiked samples were processed in the same way as real samples. The recoveries for individual analytes were calculated as [(concentration of the analyte in spiked sample − concentration in non-spiked sample)/amounts of added steroids] * 100 (%).

Matrix effect, which is the result of the ion suppression was determined according to Annesley [35] by performing post-column infusion. Visual inspection of the post-column perfusion transitions revealed no evidence of any significant drop in MS/MS signal during the retention times of measured steroids and deuterated steroids.

Carry-over was determined by running a blank solvent after the highest calibrator and by injecting a control sample of known concentration of analytes.

2.9. Data and statistical analysis

Analyst software version 1.6 (AB Sciex, Concord, Canada) was employed for data acquisition, peak-area integration and quantitation of unknown plasma and CSF samples. Calibration curves were

derived in each analytical run. Validation data were calculated using Microsoft Excel® 2013.

3. Results

3.1. Method performance characteristics

The precursor, quantification and confirmation ions of individual steroids and other optimized values as well as the retention times are summarized in Table 2. The representative chromatograms of individual steroids in plasma and cerebrospinal fluid sample are shown in Figs. 1 and 2.

The linearity of the method expressed by the correlation coefficients is summarized in Table 1. The correlation coefficients were 0.9988 and higher. The LODs and LOQs of individual analytes are summarized in Table 1. The within-day and between-day coefficients of variation (*CV*) are demonstrated in Table 1. The RSD was in all steroids lower than 15%. The recoveries in plasma and CSF samples are summarized in Table 3. The recoveries were in the range of 85–113.2% and 85.1–112.6 in plasma and CSF, respectively. In both matrices, no carry-over was detected.

3.2. Application to plasma and CSF samples

Medians and concentration ranges of individual free steroid hormones in plasma and CSF in abovementioned subjects are summarized in Table 4. There were observed no significant differences between men and women except plasma 7α-OH-DHEA; see e.g. [1].

4. Discussion

Derivatization by 2-hydrazinopyridine has been described for several steroids including DHEA, cortisol and cortisone [33,36], but so far not for 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA and 16α-OH-DHEA. These steroids are usually determined by radioimmunoassays [37–40] and by gas-chromatography–mass spectrometry [17,31,41–43]. The HPLC/MS determination for these 7-oxygenated steroids was published only for identification purposes [19,44–46].

Our LC–MS/MS method enables rapid, complex and more sensitive and precise determination of 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA and 16α-OH-DHEA, when compared to the immunoanalytical methods. Together with the determination of DHEA, cortisol and cortisone it may serve for the complex evaluation of the 11β-HSD1 activity. Considering the detection limits of each of determined steroid, using of 500 μL of plasma and 3000 μL of CSF provide satisfactory accuracy, precision and recovery.

Derivatization of analytes is effective to improve the sensitivity on LC–MS/MS and increase the detection response. Thanks to derivatization it is possible to measure abovementioned analytes in the pg/mL order of magnitude with triple stage quadrupole mass

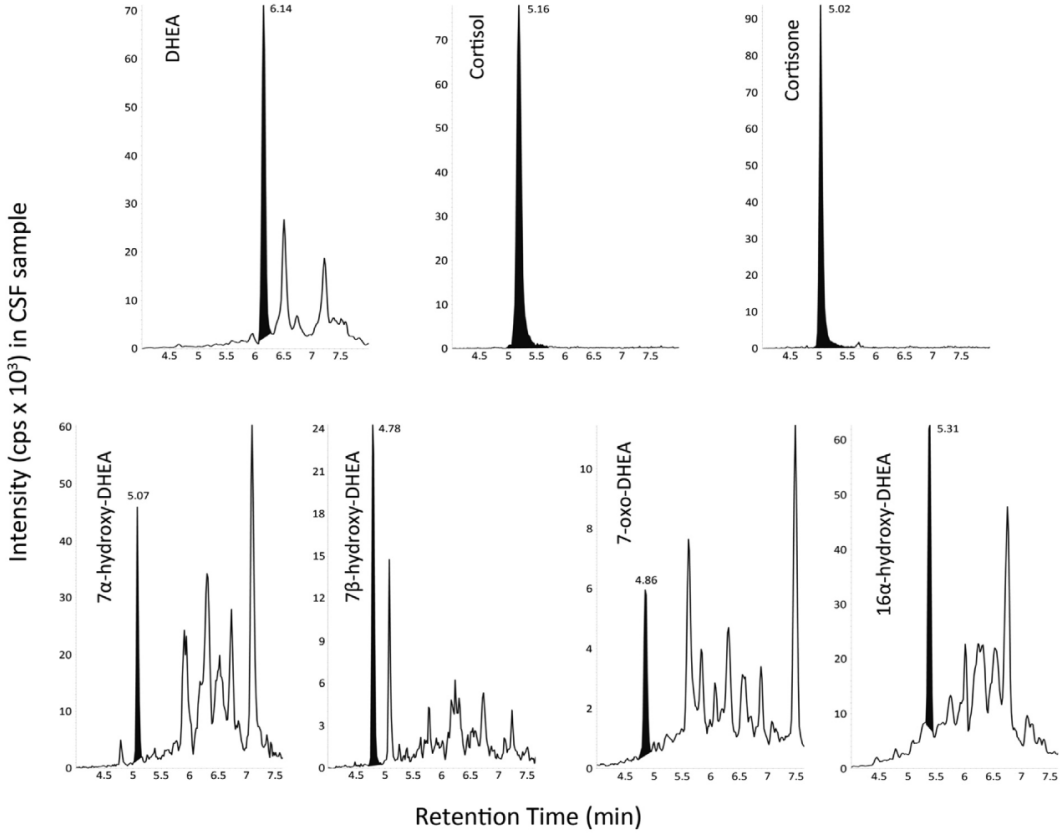


Fig. 2. Chromatograms of individual steroids in cerebrospinal fluid (CSF) sample. Transition details are presented in Table 2 and retention times (min) are indicated above the peak.

Table 3
Recovery from addition at three concentration levels in plasma.

Analyte	Amount added to plasma matrix (ng/mL)	Recovery in plasma (%)	Amount added to CSF matrix (ng/mL)	Recovery in CSF (%)
Cortisol	8	95.8	2.67	103.6
	40	92.5	10.67	96.1
	120	91.0		
Cortisone	1.6	95.8	0.67	87.1
	8	106.5	2.67	78.4
	24	110.1		
DHEA	1.6	109.7	0.027	99.4
	8	98.1	0.155	100.2
	24	93.3		
7α-OH-DHEA	0.06	101.1	0.053	108.6
	0.3	109.7	0.213	109.4
	1.5	88.4		
7β-OH-DHEA	0.06	106.1	0.027	96.5
	0.3	111.6	0.155	93.8
	1.5	85.0		
7-oxo-DHEA	0.02	104.0	0.027	89.5
	0.1	98.3	0.155	85.1
	0.5	94.2		
16α-OH-DHEA	0.02	102.5	0.027	112.6
	0.1	107.8	0.155	109.5
	0.5	113.2		

Replicate: $n = 5$ and at two concentration levels in cerebrospinal fluid (CSF), replicate: n = 5.

Table 4
Medians and concentration ranges of free steroids in plasma and cerebrospinal fluid samples. LOD means limit of detection. The concentration of steroids are provided in ng/mL.

Analyte	Plasma		Cerebrospinal fluid	
	Concentration range	Medians	Concentration range	Medians
DHEA	0.066–2.190	0.83	0.004–0.610	0.03
Cortisol	23.8–184.0	96.75	0.808–20.20	6.54
Cortisone	7.161–26.601	18.85	0.137–5.028	2.0
Cortisol/cortisone	2.72–9.02	5.06	1.15–10.31	2.91
7α-OH-DHEA	0.058–0.380	0.138	0.016–0.315	0.06
7β-OH-DHEA	0.024–0.072	0.049	0.003–0.041	0.01
7-oxo-DHEA	0.009–0.052	0.017	Under LOD	Under LOD
16α-OH-DHEA	0.027–0.068	0.051	0.005–0.027	0.006

detector. Without derivatization, more sophisticated LC–MS/MS instruments would be necessary which are not easily accessible for many research and healthcare laboratories. 500 μL of plasma is routinely used amount for the majority of medical devices. 3000 μL of CSF is a large volume, but when investigating the neurological diseases, CSF is easily accessible by performing the lumbar puncture or lumbar drainage, which is routinely carried out in the neurosurgical departments.

The analysis of CSF has become a suitable method in the diagnosis and understanding of various neurodegenerative disorders. CSF is in the close contact with the brain extracellular fluid and therefore can better reflect biological processes in the brain. Transport of the analytes from blood to CSF may be disturbed by several disorders and therefore might be CSF an excellent material in disease diagnosis [47].

To the best our knowledge, this is the first attempt to determinate free 7-oxo-DHEA in CSF at all, however the levels of this metabolite in CSF were at the edge or under the detection limit of our method (below 12 pg/mL). These results supported the hypothesis, that 7-oxo-DHEA is only an intermediate involving 7α-OH-DHEA and 7β-OH-DHEA interconversion [19]. The 11β-HSD1 activity in CSF is shifted toward hydroxylated metabolites of DHEA. This is also the first LC–MS/MS determination of free 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA, 16α-OH-DHEA in their 2-hydrazinopyridinated form.

When comparing our results in plasma and CSF samples with the results of other authors, our data from LC–MS/MS analysis are in a good agreement, especially for 7α-OH-DHEA, being very close to the data of Kancheva et al. [31,48] and Kim et al. [49], however these results show lower values than corresponding data from immunoassays [38]. It is also valid for DHEA, 7β-OH-DHEA and plasma 7-oxo-DHEA [38,39]. The lower levels obtained by instrumental methods can be explained in general by cross-reactivities and lower specificities of immunoanalytical methods. The levels of plasma 16α-OH-DHEA corresponded with both instrumental and immunoanalytical methods [31,48,50]. Its CSF levels were about three times higher when compared data from third ventricle measured by GC–MS using the single quadrupole mass spectrometer [31,48]. Concerning cortisol and cortisone in plasma and CSF, the levels were in a good agreement with one of the most recent results of Sinclair et al. [51] and McWhinney et al. [52].

The determination of the above-mentioned free steroids in plasma and CSF may help to understand of the origin of these steroids, i.e. whether they are of peripheral origin or synthesized de novo in the CNS. In general, our data are in agreement with the concept that a part of steroids may be synthesized de novo in CNS from the steroid precursors or directly transported from the periphery. The CNS synthesis and transport from the periphery might be complementary in some cases, i.e. brain synthesis might provide minimum level of steroids, which are indispensable for the CNS functions [31].

Our and other author's results [31] indicate relatively close concentrations of free 7α-OH-DHEA in CSF and plasma. These results demonstrated relatively uncomplicated transport of this steroid between CSF and peripheral circulation and supported the concept that 7α-OH-DHEA may originate in the brain and also penetrate from the periphery in the CNS. 7α-OH-DHEA is in CNS further converted to 7β-OH-DHEA via 7-oxo-DHEA in the CNS. Among free 7α-OH-DHEA, we found substantially lower levels of the CNS steroids in comparison with the levels in circulation. Free cortisol and cortisone levels in CSF were lower than in plasma, which is in accordance with other author's results [51]. 11βHSD1 is expressed in humans in many brain areas [53] suggesting that 11βHSD1 could be an important regulator of the local *(in situ)* concentration of biologically active hormone. With respect to its counterregulatory potential to 7-oxygenated steroids, the determination of free 16α-OH-DHEA in CSF may serve as additional marker of brain disorders of autoimmune origin. As with the majority of determined steroids, the levels of free 16α-OH-DHEA were also lower in CSF compared to the levels in plasma.

5. Conclusion

The standardized sample preparation procedure, detection limits, analysis time and the multi-analyte estimation make this LC–MS/MS method suitable for the determination of free DHEA and its 16α-OH-, 7α-OH-, 7β-OH- and 7-oxo-metablites. This combination of these analytes together with free cortisol and cortisone enables the estimation of 11β-HSD1 activity in plasma and CSF. The simultaneous determination of the abovementioned free steroids in plasma and CSF may be helpful for better understanding of the development and progression of various neurodegenerative

diseases including Alzheimer's dementia and normal pressure hydrocephalus.

Acknowledgement

This work was supported by Grants IGA NT12349-4 and NT13369-4 from the Czech Ministry of Health.

References

[1] Hampl R, Hill M, Stárka L. DHEA metabolites during the life span. In: Morfin R, editor. DHEA and the brain. London and New York: Taylor & Francis; 2003.
[2] El Kihel L. Oxidative metabolism of dehydroepiandrosterone (DHEA) and biologically active oxygenated metabolites of DHEA and epiandrosterone (EpiA)-recent reports. Steroids 2012;77:10–26.
[3] Doostzadeh J, Cotillon A-C, Benalychérif A, Morfin R. Inhibition studies of dehydroepiandrosterone 7α- and 7β-hydroxylation in mouse liver microsomes. Steroids 1998;63:608–14.
[4] Doostzadeh J, Morfin R. Studies of the enzyme complex responsible for pregnenolone and dehydroepiandrosterone 7α-hydroxylation in mouse tissues. Steroids 1996;61:613–20.
[5] Akwa Y, Morfin RF, Robel P, Baulieu EE. Neurosteroid metabolism. 7 alpha-hydroxylation of dehydroepiandrosterone and pregnenolone by rat brain microsomes. Biochem J 1992;288(Pt 3):959–64.
[6] Okada M, Fukushima DK, Gallagher TF. Isolation and characterization of 3 beta-hydroxy-delta 5-steroids in adrenal carcinoma. J Biol Chem 1959;234:1688–92.
[7] Stárka L. 7α-Hydroxylierung von dehydroepiandrosteron in menschlicher nebennierenrinde und leber. Naturwissenschaften 1965;52:499.
[8] Morfin R, Lafaye P, Cotillon AC, Nato F, Chmielewski V, Pompon D. 7a-Hydroxy-dehydroepiandrosterone and immune response. Annals NY Acad Sci 2000;917:971–82.
[9] Hampl R, Hill M, Šterzl I, Starka L. Immunomodulatory 7-hydroxylated metabolites of dehydroepiandrosterone are present in human semen. J Steroid Biochem Mol Biol 2000;75:273–6.
[10] Pelissier MA, Trap C, Malewiak MI, Morfin R. Antioxidant effects of dehydroepiandrosterone and 7alpha-hydroxy-dehydroepiandrosterone in the rat colon, intestine and liver. Steroids 2004;69:137–44.
[11] Akwa Y, Young J, Kabbadj K, Sancho MJ, Zucman D, Vourc'h C, et al. Neurosteroids: biosynthesis, metabolism and function of pregnenolone and dehydroepiandrosterone in the brain. J Steroid Biochem Mol Biol 1991;40:71–81.
[12] Jellinck PH, Croft G, McEwen BS, Gottfried-Blackmore A, Jones G, Byford V, et al. Metabolism of dehydroepiandrosterone by rodent brain cell lines: relationship between 7-hydroxylation and aromatization. J Steroid Biochem Mol Biol 2005;93:81–6.
[13] Morfin R, Courchay G. Pregnenolone and dehydroepiandrosterone as precursors of native 7-hydroxylated metabolites which increase the immune response in mice. J Steroid Biochem Mol Biol 1994;50:91–100.
[14] Fukushima DK, Kemp AD, Schneider R, Stokem MB, Gallagher TF. Studies in steroid metabolism. XXV. Isolation and characterization of new urinary steroids. J Biol Chem 1954;210:129–37.
[15] Lardy H, Partridge B, Kneer N, Wei Y. Ergosteroids: induction of thermogenic enzymes in liver of rats treated with steroids derived from dehydroepiandrosterone. Proc Natl Acad Sci USA 1995;92:6617–9.
[16] Bobyleva V, Bellei M, Kneer N, Lardy H. The effects of the ergosteroid 7-oxo-dehydroepiandrosterone on mitochondrial membrane potential: possible relationship to thermogenesis. Arch Biochem Biophys 1997;341:122–8.
[17] Hill M, Havlikova H, Vrbikova J, Kancheva R, Kancheva L, Pouzar V, et al. The identification and simultaneous quantification of 7-hydroxylated metabolites of pregnenolone, dehydroepiandrosterone, 3beta,17beta-androstenediol, and testosterone in human serum using gas chromatography–mass spectrometry. J Steroid Biochem Mol Biol 2005;96:187–200.
[18] Gottfried-Blackmore A, Jellinck PH, Vecchiarelli HA, Masheeb Z, Kaufmann M, McEwen BS, et al. 7alpha-hydroxylation of dehydroepiandrosterone does not interfere with the activation of glucocorticoids by 11beta-hydroxysteroid dehydrogenase in E(t)C cerebellar neurons. J Steroid Biochem Mol Biol 2013;138:290–7.
[19] Muller C, Pompon D, Urban P, Morfin R. Inter-conversion of 7alpha- and 7beta-hydroxy-dehydroepiandrosterone by the human 11beta-hydroxysteroid dehydrogenase type 1. J Steroid Biochem Mol Biol 2006;99:215–22.
[20] Ferroud C, Revial G, Morfin R. Chemical and biochemical approaches to the production of 7-hydroxylated C19-steroids. Horm Mol Biol Clin Invest 2012;10:293–9.
[21] Robinzon B, Michael KK, Ripp SL, Winters SJ, Prough RA. Glucocorticoids inhibit interconversion of 7-hydroxy and 7-oxo metabolites of dehydroepiandrosterone: a role for 11beta-hydroxysteroid dehydrogenases? Arch Biochem Biophys 2003;412:251–8.
[22] Seckl JR, Walker BR. 11beta-hydroxysteroid dehydrogenase type 1 as a modulator of glucocorticoid action: from metabolism to memory. Trends Endocrinol Med 2004;15:418–24.
[23] Nashev LG, Chandsawangbhuwana C, Balazs Z, Atanasov AG, Dick B, Frey FJ, et al. Hexose-6-phosphate dehydrogenase modulates 11beta-hydroxysteroid dehydrogenase type 1-dependent metabolism of 7-keto- and 7beta-hydroxy-neurosteroids. PLoS One 2007;2:e561.
[24] Fotherby K, Colas A, Atherden SM, Marrian GF. The isolation of 16alpha-hydroxydehydroepiandrosterone (3beta:16alpha-dihydroxyandrost-5-en-17-one) from the urine of normal men. Biochem J 1957;66:664–9.
[25] Reynolds JW. The excretion of two delta-5-3-beta-OH, 16-alpha-hydroxysteroids by patients with congenital adrenal hyperplasia. Pediatrics 1965;36:583–91.
[26] Cleary RE, Pion RJ. Urinary excretion of 16 alpha-hydroxydehydroepiandrosterone and 16-ketoandrostenediol during the early neonatal period. J Clin Endocr Metab 1968;28:372–8.
[27] Tagawa N, Kusuda S, Kobayashi Y. C16 hydroxylation of 3beta-hydroxy-delta5-steroids during the early neonatal period. Biol Pharm Bull 1997;20:1295–9.
[28] Heřt J, Hill M, Hampl R. Gas chromatographic–mass spectrometric identification of 16α-hydroxy-dehydroepiandrosterone in human seminal plasma. Steroids 2004;69:773–7.
[29] Pouzar V, Černý I, Hill M, Bičíková M, Hampl R. Derivatives of 16α-hydroxy-dehydroepiandrosterone with an additional 7-oxo or 7-hydroxy substituent: synthesis and gas chromatography/mass spectrometry analysis. Steroids 2005;70:739–49.
[30] Hampl R, Starka L. Minireview: 16α-hydroxylated metabolites of dehydroepiandrosterone and their biological significance. Endocr Regul 2000;34:161–3.
[31] Kancheva R, Hill M, Novak Z, Chrastina J, Velikova M, Kancheva L, et al. Peripheral neuroactive steroids may be as good as the steroids in the cerebrospinal fluid for the diagnostics of CNS disturbances. J Steroid Biochem Mol Biol 2010;119:35–44.
[32] Walchenbach R, Geiger E, Thomeer RTWM, Vanneste JAL. The value of temporary external lumbar CSF drainage in predicting the outcome of shunting on normal pressure hydrocephalus. J Neurol Neurosurg Psychiatry 2002;72:503–6.
[33] Higashi T, Nishio T, Hayashi N, Shimada K. Alternative procedure for charged derivatization to enhance detection responses of steroids in electrospray ionization-MS. Chem Pharm Bull 2007;55:662–5.
[34] Taverniers I, De Loose M, Van Bockstaele E. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. TrAC 2004;23:535–52.
[35] Annesley TM. Ion suppression in mass spectrometry. Clin Chem 2003;49:1041–4.
[36] Higashi T, Shibayama Y, Shimada K. Determination of salivary dehydroepiandrosterone using liquid chromatography–tandem mass spectrometry combined with charged derivatization. J Chromatogr B Anal Technol Biomed Life Sci 2007;846:195–201.
[37] Lapcik O, Hampl R, Hill M, Bičíková M, Starka L. Immunoassay of 7-hydroxysteroids: 1. Radioimmunoassay of 7b-hydrxoy dehydroepiandrosterone. J Steroid Biochem Mol Biol 1998;67:439–45.
[38] Lapcik O, Hampl R, Hill M, Starka L. Immunoassay of 7-hydroxysteroids: 1. Radioimmunoassay of 7a-hydrxoy dehydroepiandrosterone. J Steroid Biochem Mol Biol 1999;71:231–7.
[39] Kazihnitkova H, Zamrazilova L, Hill M, Lapcik O, Pouzar V, Hampl R. A novel radioimmunoassay of 7-oxo-DHEA and its physiological levels. Steroids 2007;72:342–50.
[40] Hampl R, Hill M, Starka L. 7-Hydroxydehydroepiandrosterone epimers in the life span. J Steroid Biochem Mol Biol 2001;78:367–72.
[41] Hill M, Lapcik O, Havlíková H, Morfin R, Hampl R. 7-Hydroxydehydroepiandrosterone epimers in human serum and saliva comparison of gas chromatography–mass spectrometry and radioimmunoassay. J Chromatogr A 2001;935:297–307.
[42] Matsuzaki Y, Yoshida S, Honda A, Miyazaki T, Tanaka N, Takagiwa A, et al. Simultaneous determination of dehydroepiandrosterone and its 7-oxygenated metabolites in human serum by high-resolution gas chromatography–mass spectrometry. Steroids 2004;69:817–24.
[43] Robinzon B, Miller KK, Prough RA. Biosynthesis of [3H]7 alpha-hydroxy-, 7beta-hydroxy-, and 7-oxo-dehydroepiandrosterone using pig liver microsomal fractions. Anal Biochem 2004;333:128–35.
[44] Marwah A, Gomez FE, Marwah P, Ntambi JM, Fox BG, Lardy H. Redox reactions of dehydroepiandrosterone and its metabolites in differentiating 3T3-L1 adipocytes: a liquid chromatographic-mass spectrometric study. Arch Biochem Biophys 2006;456:1–7.
[45] Jellinck PH, Kaufmann M, Gottfried-Blackmore A, Croft G, Byford V, McEwen BS, et al. Dehydroepiandrosterone (DHEA) metabolism in the brain: identification by liquid chromatography/mass spectrometry of the delta-4-isomer of DHEA and related steroids formed from androstenedione by mouse BV2 microglia. J Steroid Biochem Mol Biol 2006;98:41–7.
[46] Chalbot S, Morfin R. Human liver S9 fractions: metabolism of dehydroepiandrosterone, epiandrosterone, and related 7-hydroxylated derivatives. Drug Metab Dispos 2005;33:563–9.
[47] Stoop MP, Coulier L, Rosenling T, Shi S, Smolinska AM, Buydens L, et al. Quantitative proteomics and metabolomics analysis of normal human cerebrospinal fluid samples*. Mol Cell Proteomics 2010;9:2063–75.
[48] Kancheva R, Hill M, Novak Z, Chrastina J, Kancheva L, Starka L. Neuroactive steroids in periphery and cerebrospinal fluid. Neuroscience 2011;191:22–7.
[49] Kim SB. Neurosteroids: cerebrospinal fluid levels for Alzheimer's disease and vascular dementia diagnostics. J Clin Endocrinol Metab 2003;88:5199–206.

[50] Zamrazilová L, Kazihnitková H, Lapčík O, Hill M, Hampl R. A novel radioimmunoassay of 16α-hydroxy-dehydroepiandrosterone and its physiological levels. J Steroid Biochem Mol Biol 2007;104:130–5.
[51] Sinclair AJ, Walker EA, Burdon MA, van Beek AP, Kema IP, Hughes BA, et al. Cerebrospinal fluid corticosteroid levels and cortisol metabolism in patients with idiopathic intracranial hypertension: a link between 11beta-HSD1 and intracranial pressure regulation? J Clin Endocrinol Metab 2010; 95:5348–56.
[52] McWhinney BC, Briscoe SE, Ungerer JP, Pretorius CJ. Measurement of cortisol, cortisone, prednisolone, dexamethasone and 11-deoxycortisol with ultra high performance liquid chromatography–tandem mass spectrometry: application for plasma, plasma ultrafiltrate, urine and saliva in a routine laboratory. J Chromatogr B 2010;878:2863–9.
[53] Bisschop PH, Dekker MJ, Osterthun W, Kwakkel J, Anink JJ, Boelen A, et al. Expression of 11beta-hydroxysteroid dehydrogenase type 1 in the human hypothalamus. J Neuroendocrinol 2013;25:425–32.

PŘÍLOHA VIII

Vitku J, Chlupacova T, Sosvorova L, Hampl R, Hill M, Heracek J, Bicikova M, Starka L: Development and validation of LC-MS/MS method for quantification of bisphenol A and estrogens in human plasma and seminal fluid. Talanta, 140, 62-7 (2015) IF = 3.545

Talanta 140 (2015) 62–67

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development and validation of LC–MS/MS method for quantification of bisphenol A and estrogens in human plasma and seminal fluid

Jana Vitku [a,*], Tereza Chlupacova [a], Lucie Sosvorova [a], Richard Hampl [a], Martin Hill [a], Jiri Heracek [b,c,d], Marie Bicikova [a], Luboslav Starka [a]

[a] Institute of Endocrinology, Department of Steroids and Proteofactors, Narodni 8, 116 94 Prague, Czech Republic
[b] Charles University in Prague, Department of Urology, Šrobárova 50, 100 34 Prague, Czech Republic
[c] Faculty Hospital Kralovske Vinohrady, Department of Urology, Šrobárova 50, 100 34 Prague, Czech Republic
[d] Military University Hospital Prague, Department of Urology, U Vojenske nemocnice 1200, 169 02 Prague, Czech Republic

ARTICLE INFO

Article history:
Received 3 February 2015
Received in revised form
3 March 2015
Accepted 6 March 2015
Available online 12 March 2015

Keywords:
Bisphenol A
Estrone
Estradiol
Estriol
Semen
LC–MS

ABSTRACT

Bisphenol A (BPA) is a widely known endocrine disruptor with estrogenic, antiestrogenic or anti-androgenic properties. BPA could interfere with estrogen metabolism as well with receptor-mediated estrogen actions. Both environmental BPA and estrogens may be traced in body fluids, of which, besides the blood plasma, the seminal fluid is of particular interest regarding their possible interactions in the testis. The method for simultaneously determining BPA and estrogens is then needed, taking into account that their concentrations in these body fluid may differ. Here the method was developed and validated for measurements of BPA, estrone (E1), estradiol (E2) and estriol (E3) in blood plasma and seminal plasma using liquid chromatography–tandem mass spectrometry. Due to the phenolic moiety of all compounds, dansyl chloride derivatization could be used. The analytical criteria of the method with respect to expected concentration of the analytes were satisfactory. The lower limits of quantifications (LLOQ) amounted to 43.5, 4.0, 12.7, 6.7 pg/mL for plasma BPA, E1, E2 and E3, and 28.9, 4.9, 4.5, 3.4 pg/mL for seminal BPA, E1, E2 and E3, respectively. The concentrations of individual steroids differed between body fluids. To the best of our knowledge, this is the first method that enabled the measurement of estrogens and BPA together in one run. The concentrations of E1, E2 and for the first time also of E3 in seminal plasma in normospermic men are reported.

1. Introduction

In recent decades, a large amount of chemicals has been introduced to the environment by anthropogenic activities. Many of them interfere with the endocrine system as so called endocrine disruptors (EDs). Bisphenol A (BPA) is one of the known EDs, which is widely used in polycarbonate and other plastics, epoxy resins, dental sealants, consumer electronics or thermal receipts [1,2]. BPA exposure has been associated with a variety of health complications including obesity, type 2 diabetes, cardiovascular disease and reproductive disorders [2,3]. Specifically in men, the relationship between BPA levels and decreased semen quality, sperm DNA damage and changes in reproductive hormones have been reported [4–7].

Multiple studies reported the determination of unconjugated BPA in serum/plasma (reviewed in [1,2]). However, a discussion has been raised about potential external contamination with BPA and method sensitivities. Therefore additional studies are needed to accurately determine BPA exposure in the general population [3]. A detailed evaluation of formerly published methods for measuring BPA in serum/plasma was reviewed by Vom Saal and Welshons [1]. On the other hand, the knowledge about BPA in seminal plasma is limited.

Research addressing the impact of BPA on steroidogenesis in men used BPA measurements in urine [7–10]. The results indicate that BPA can alter the steroid hormone pathway in men, although most of the studies dealt with infertile men. Therefore, more data are necessary to appraise the exact effects of BPA on steroidogenesis, especially in fertile men [7].

BPA possess a weak estrogenic activity similarly to endogenous estrogens, but it may also act as antiestrogen or antiandrogen [3,11,12]. Taken together, BPA effects are very complex and wide-ranging (for review see [3]). From a chemical point of view, estrogens and BPA possess phenolic moiety, therefore, it offers the

* Corresponding author. Tel.: +420 224 905 238.
E-mail addresses: jvitku@endo.cz (J. Vitku), tchlupacova@endo.cz (T. Chlupacova), lsosvorova@endo.cz (L. Sosvorova), rhampl@endo.cz (R. Hampl), mhill@endo.cz (M. Hill), jiri.heracek@lf1.cuni.cz (J. Heracek), mbicikova@endo.cz (M. Bicikova), lstarka@endo.cz (L. Starka).

http://dx.doi.org/10.1016/j.talanta.2015.03.013
0039-9140/

possibility for dansyl chloride derivatization. In addition, no study has reported the simultaneous determination of estrogens and BPA together, which would be useful in discovering the effects of BPA on estrogen metabolism.

The aim of the study was to develop with sufficient accuracy and sensitivity the LC–MS/MS assay for the simultaneous determination of unconjugated bisphenol A and estrogens (estrone, estradiol, estriol) in human plasma and seminal plasma. Application of this method allowed for the measurement of analytes in both matrices in 79 normospermic men. The LC–MS/MS assay was compared with the GC–MS method to determine E2 as well as with radioimmunoassay (RIA).

2. Experimental

2.1. Reagents and materials

The steroids estrone (E1), 17β-estradiol (E2) and estriol (E3) and deuterated standards of estrone (d4E1) and estriol (d2E3) were purchased from Steraloids (Newport, RI, USA). Bisphenol A (BPA), deuterated BPA (d16BPA) and deuterated E2 (d3E2) were obtained from Sigma-Aldrich (St. Louis, MO, USA) as well as 99.9% tert-butyl methyl ether (MTBE), acetone, sodium bicarbonate, sodium hydroxide and dansyl chloride. Methanol and water for chromatography were purchased from Merck (Darmstadt, Germany). All solvents and reagents were of HPLC grade.

2.2. Preparation of reagents

The sodium bicarbonate buffer (100 mM, pH 10.5) was prepared by dissolving 0.42 g sodium bicarbonate in 50 mL of ultrapure water. The pH was adjusted to 10.5 with aqueous 1 M sodium hydroxide.

Physiological solution was prepared by adding 4 g of sodium chloride to 0.5 L of ultrapure water to give 0.9% solution.

2.3. Preparation of stock solutions, working standard solutions and calibration mixtures

Stock (1 mg/mL) and working solutions (1 μg/mL) in methanol were prepared for each compound and stored at −20 °C. The calibration mixture was prepared from the individual working solutions in a concentration of 4 ng/mL for BPA and 1 ng/mL for estrogens. The mixture of internal standards (IS) in methanol was prepared similarly to give final concentrations of 100 ng/mL for d16BPA and 50 ng/mL for estrogens. Mixtures were stored at −20 °C. Eight point calibration curves were constructed in the range of 0.008–1 ng/mL for estrogens and 0.032–4 ng/mL for BPA for both matrices.

2.4. Samples

Samples of plasma and seminal plasma were obtained from patients attending the Center of Assisted Reproduction Pronatal (Prague, CZ). Each patient underwent standardized ejaculate examination (spermiogram) according to the World Health Organization (WHO) criteria. The group of 79 patients was specified by reproductive age (35.8 ± 4.7 years) with normospermic spermiogram. The protocol was approved by the Ethical Committee of the Institute of Endocrinology. Informed and written consent with the use of biological materials for research reasons was obtained from all subjects participating to the project. All samples were stored at −20 °C.

2.5. Extraction, derivatization and optimization

A sample of plasma (500 μL) or seminal plasma (1000 μL) was spiked with 10 μL of IS mixture and diluted with 500 μL of physiological solution. Samples were shaken and liquid–liquid extraction (LLE) using MTBE (2 mL, 1 min) was performed. The organic phase was transferred to clean glass tube and evaporated until dryness using a vacuum concentrator (55 °C).

The derivatization step was performed according to Anari et al. [13] with certain modifications. A volume of 50 μL of bicarbonate buffer (100 mM, pH 10.5) and 50 μL of dansyl chloride in acetone (1 mg/mL) was added to the dry residues and shortly vortexed. The mixture was incubated at 60 °C for 5 min and then let cool down to room temperature. Thereafter, the samples were evaporated to dryness using vacuum concentrator (55 °C). The dry residues were reconstituted with 300 μL of methanol and 50 μL of the solution was transferred to the vial with a glass insert where 50 μL of the ammonium formate in ultrapure water (10 mM) was pre-pipetted. The volume of 50 μL of the sample was injected into LC–MS/MS for analysis.

Different reagents for extraction were tested (MTBE vs diethyl ether) and also various volumes of extraction agents were tried out (1 mL, 2 mL and 3 mL for both reagents). The best recovery was obtained when 2 mL of MTBE was used.

2.6. Liquid chromatography

The ultra-high performance liquid chromatography (UHPLC) Eksigent ultraLC 110 system (Redwood City, CA, USA) equipped with a Kinetex C18 column (100 mm × 3.0 mm, 1.7 μm; Phenomenex, Torrance, CA, USA) and Security Guard ULTRA cartridge system (UHPLC C18 for 3 mm ID column; Phenomenex, Torrance, CA, USA) was used for the analysis. Column temperature was maintained at 50 °C and separation was carried out at a flow rate of 0.4 mL/min.

HPLC grade water (A) and methanol (B) were used as mobile phases. A gradient elution started at 50% B (0–2 min); linearly increased to 90% B (2–6 min), then to 95% B (6–7 min), maintained

Table 1
Retention times, precursor ions, fragment ions and MS optimized conditions (declustering potential, DP; entrance potential, EP; collision entrance potential, CEP; collision energy, CE; collision cell exit potential, CXP) for all analytes.

Analyte	Retention time (min)	Precursor ion	Quantification ion	Confirmation ion	DP (V)	EP (V)	CEP (V)	CE (V)[a]	CXP (V)
BPA	9.31	695.24	171.04	170.05	86	6.5	28	65 (65)	4
d16BPA	9.25	709.28	171.11	170.15	86	7.5	28	65 (65)	4
E1	8.07	504.18	171.00	156.01	71	5	22	47 (75)	4
d4E1	8.06	508.23	171.09	156.07	71	5	24	49 (77)	4
E2	8.26	506.17	170.98	155.98	76	5	24	49 (75)	4
d3E2	8.24	509.24	171.09	156.09	76	5	22	49 (73)	4
E3	7.29	522.24	171.03	156.02	76	5	22	49 (79)	4
d2E3	7.28	524.27	171.10	156.10	71	8	24	47 (77)	4

[a] Values for the confirmation ion are given in the brackets.

at 95% B (7–8.8 min), dropped to 50% B (8.8–9 min) and stayed at 50% B from 9 min to 11 min. Retention times of the analytes are given in Table 1.

2.7. Mass spectrometry

Detection of the analytes was performed on an API 3200 mass spectrometer (AB Sciex, Concord, Canada) with electrospray ionization (ESI) probe operating in a positive mode. Ion source and MS/MS conditions were optimized by infusion of 0.2 μg/mL of the individual derivatized analytes to MS at 20 μL/min. The optimal conditions were as follows: ion spray voltage of 5500 V, temperature of 600 °C, curtain gas of 25.0 psi (172.38 kPa), collision gas of 4 psi (25.58 kPa), ion source gas 1 of 40.0 psi (275.79 kPa) and ion source gas 2 of 60.0 psi (413.69 kPa). Ions were examined in multiple reaction monitoring mode (MRM). Transitions with optimized conditions for MS are listed in Table 1. Analyst 1.6 software was used for system control and data evaluation.

2.8. Validation

The analytical method was validated according to FDA Guidance for Industry [14]. Validation parameters include (1) selectivity, (2) precision, (3) recovery (analytical accuracy), (4) calibration curve, and (5) stability of the analytes in spiked samples.

Acceptable selectivity was defined as the absence of any detectable SRM LC–MS/MS ion currents at the retention time regions of each analyte and its deuterated standards in blank plasma samples (double blanks).

Accuracy, precision and recovery were determined by using 6 samples per concentration; four different concentrations were assessed for plasma analytes and three different concentrations were examined for seminal fluid analytes. Pooled plasma samples or pooled seminal fluid containing IS were used as the first concentration. The spiked concentrations were as follows: 0.02, 0.1, 0.24 ng/mL for plasma estrogens (E1, E2, E3); 0.08, 0.40, 0.96 ng/mL for plasma BPA; 0.04 and 0.2 for seminal fluid estrogens and 0.16 and 0.8 ng/mL for seminal fluid BPA. Samples were pretreated in the same way as in Section 2.5.

Intra-assay precision (repeatability) and inter-assay precision (intermediate precision) were assessed and the values are expressed as relative standard deviation (RSD).

The recoveries for individual analytes were calculated as [(concentration of the analyte in spiked sample − concentration in non-spiked sample)/amounts of added steroids] × 100 (%).

Lower limit of quantification (LLOQ) was defined as 10 × standard deviation/slope of the calibration curve.

Freeze and thaw stability test, short-term temperature stability test, long-term stability test, stock solution stability test and post-preparative stability test were examined.

3. Results

3.1. Selectivity, precision and recovery

There were no detectable SRM LC–MS/MS currents at the retention time regions of all analytes. A satisfactory assay accuracy (analytical recovery) ranging from 92.3% to 104.0% for plasma and 95.0% to 103.8% for seminal fluid was obtained. Precision did not exceed 15% of the RSD at all concentrations in both matrices. The results are given in Tables 2 and 3 for plasma and seminal fluid, respectively.

Table 2
Validation parameters for analytes in plasma.

Plasma		Precision (%)		Recovery (%)
Compound	Added (ng/ml)	Intra-day	Inter-day	
BPA	0	11.55	8.8	
	0.08	10.57	7.6	93.7
	0.4	7.86	3.5	92.3
	0.96	8.69	8.3	103.0
E2	0	8.23	2.2	
	0.02	8.7	0.5	97.7
	0.1	5.92	5.1	103.2
	0.24	6.05	5	98.1
E1	0	10.99	8.4	
	0.02	7.12	6	103.4
	0.1	8.92	2.9	102.0
	0.24	5.77	7	103.3
E3	0	10.98	14.3	
	0.02	8.86	9.4	99.6
	0.1	7.35	3.4	104.0
	0.24	5.57	3.1	102.0

Table 3
Validation parameters for analytes in seminal fluid.

Seminal fluid		Precision (%)		Recovery (%)
Compound	Added (ng/ml)	Intra-day	Inter-day	
BPA	0	9.25	6.6	
	0.16	6.89	6.5	99.7
	0.8	5.36	1.7	103.8
E2	0	13.96	3.2	
	0.04	8.82	3.3	96.1
	0.2	5.61	6.6	98.6
E1	0	12.21	11.1	
	0.04	8.02	5.6	95
	0.2	4.27	2.9	96.7
E3	0	12.46	6.9	
	0.04	5.27	6.2	100.7
	0.2	4.14	2.4	99.8

3.2. Calibration curve and matrix effect

Charcoal-treated plasma was used as blank matrix for plasma estrogens calibration curve. The plasma contained a small amount of BPA, apparently during the manufacturing process, therefore it could not be used for preparation of the calibration curves for BPA in plasma. Hence, we examined five sets of calibration curves containing IS in charcoal-treated plasma and five sets of calibrations in physiological solution similarly as Higashi et al. [15]. The regression lines were constructed with $1/x$ weighting and reached good linearity ($r > 0.9991$) and acceptable reproducibility [0.567 ± 0.0645 (mean ± SD) and 11.39% (RSD)]. Slopes of the lines obtained from physiological solution did not significantly differ from slopes from standard-added plasma.

Similarly, we compared the slopes of calibration curves prepared in physiological solution and pooled seminal plasma due to the impossibility to produce or buy the seminal plasma stripped of steroids and BPA. BPA calibration prepared in a physiological solution showed good linearity ($r > 0.9990$] and reproducibility

[1.2040 ± 0.0585 (mean ± SD) and 4.9% (RSD)]. Slopes showed no significant difference and we therefore concluded that plasma and seminal plasma matrix have no significant impact on BPA determination.

Calibration curves for estrogens constructed in a physiological solution exhibited good linearity with satisfactory correlation coefficients ($r > 0.9991$ for each estrogen) and reproducibility of slopes [2.19 ± 0.1511 (mean ± SD) and 6.9% (RSD) for E2; 2.15 ± 0.1196 and 5.6% for E1; 2.01 ± 0.18 and 8.8% for E3]. Slopes of calibration curves were compared in the same manner as BPA with slopes of calibration curves constructed in pooled seminal plasma. There was no significant difference observed for any estrogen. Therefore, seminal plasma did not display any matrix effect for estrogen determination.

Calibration ranges and LLOQs for each steroid are provided in Table 4.

3.3. Stability tests

3.3.1. Freeze and thaw stability test

We evaluated the stability of the samples after three freeze and thaw cycles. Three aliquots of each of the low (0.05 ng/mL and 0.2 ng for estrogens and BPA, respectively) and high (0.6 ng/mL and 2.4 ng/mL for estrogens and BPA, respectively) concentrations of plasma were used. Due to the limited amount of seminal fluid, we used two aliquots of low concentration (0.125 ng/mL for BPA, 0.005 ng/mL for E1, 0.007 ng/mL for E2 and 0.03 for E3) and two aliquots of high concentration (1.26 ng/mL for BPA, 0.285 ng/mL for E1, 0.287 ng/mL for E2 and 0.31 ng/mL for E3). After performing the tests, there were no statistical differences between analyte concentrations in these groups.

3.3.2. Short-term temperature stability

We evaluated short-term temperature test with the same set of sample concentrations as in freeze and thaw stability test. After 19 h in laboratory temperature, the samples were analyzed and compared with samples, which were immediately processed after thawing. We did not observe any statistical differences between analyte concentrations in these groups.

Table 4
Calibration ranges and lower limits of quantifications of all analytes in plasma and seminal fluid.

Analyte	Calibration range (ng/ml)	Seminal fluid LLOQ (pg/ml)	Plasma LLOQ (pg/ml)
BPA	0.032–4	28.9	43.5
E2	0.008–1	4.9	4.0
E1	0.008–1	4.5	12.7
E3	0.008–1	3.4	6.7

3.3.3. Long-term stability testing

Charcoal treated plasma with two different concentrations added (0.2 and 2.4 ng/mL for BPA and 0.05 and 0.8 ng/mL for estrogens, respectively) was prepared at the beginning of the validation tests and aliquoted. These samples were used in duplicates through all validation experiments and also for measuring plasma samples from normospermic men. Peak intensities of the samples were monitored continuously. Concentrations of the samples were compared with the appropriate concentrations from the first day of testing. The values in each concentration did not differ significantly.

Similar results were observed for seminal fluid samples. Pooled seminal fluid was used as the low concentration (0.125 ng/mL for BPA, 0.005 ng/mL for E1, 0.007 ng/mL for E2 and 0.03 ng/mL for E3) and the pooled seminal fluid with the addition of appropriate standards was used as the high concentration (2.4 ng/mL for BPA, 0.57 ng/mL for E1 and E2, 0.60 ng/mL for E3).

3.3.4. Stock solution stability

The stability of stock solutions of analytes and deuterated standards was evaluated after 15 h at room temperature. Two calibration curves were prepared using the stock solution left in a room temperature for the relevant period and subsequently compared by two calibration curves constructed from freshly prepared solutions. The instrument responses remained unchanged.

3.3.5. Post-preparative stability

The stability of samples was assessed after 24 h of remaining in the autosampler. The test showed that instrument responses of the samples remain unchanged at least for 1 day.

3.4. Comparison of the method with gas chromatography–mass spectrometry (GC–MS) method and radioimmunoassay (RIA)

We compared E2 in 20 plasma samples measured by the present method and the published GC–MS method [16]. In addition, plasma E2 was compared with the commercial RIA kit from Cisbio Bioassays. Both reference methods showed strong correlations with the present method ($r=0.93$ and $r=0.90$ for GC–MS and RIA respectively). Regression showed the equations $y=1.04x - 0.004$ and $y=0.2x+0.004$ for comparison with GC–MS and RIA, respectively. Plots of fitted models are shown in Fig. 1. The slope of the second regression analyzes indicated considerable overestimation by RIA.

3.5. Determination of estrogens and BPA in plasma and seminal fluid in normospermic men

Plasma BPA, E2, E1 and E3 were detected in 73%, 90%, 100% and 82% samples, respectively. Seminal BPA, E2, E1 and E3 were detected

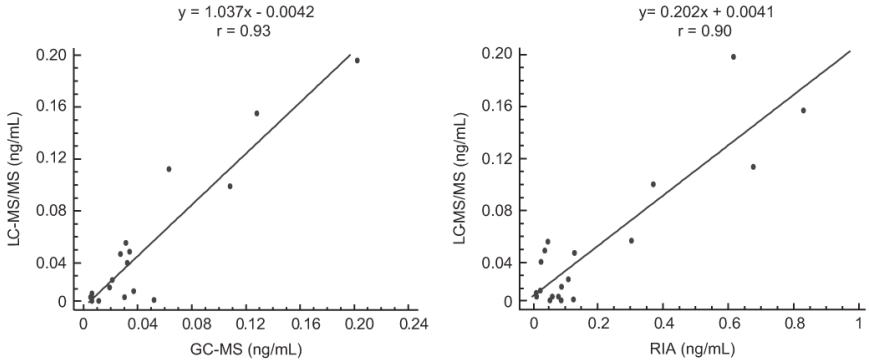


Fig. 1. Simple regression for E2. GC–MS and RIA, respectively, were selected as reference methods (x) and LC–MS/MS as a test method (y).

in 87%, 72%, 85% and 99% samples, respectively. Concentration ranges of each analytes and medians of measured samples in both fluids are given in Table 5. Examples of chromatograms obtained from the samples are shown in Fig.2.

All steps in protocol were performed using glass equipment e.g. Pasteur pipettes, glass syringes and glass tubes and controlled for contamination. Collection tubes were the only plastic that the blood came into contact with. The dwell time at collection tubes was reduced to the minimum. Nevertheless, we tested whether it could lead to contamination. Plasma collection tubes with K2EDTA did not display BPA contamination. Surprisingly, collection tubes for serum appeared to be a significant source of BPA. We could hypothesize that this is a consequence of BPA leakage from the separator gel. We therefore used collection tubes with K2EDTA. Total BPA contamination was below the limit of detection.

4. Discussion

Sex steroids (androgens and estrogens) are not only synthesized in the testis, but also act there through their receptors in both testicular cell types [17]. In the seminal fluid, in concert with its other constituents, they participate in maintainance of a unique milieu for protection and maturation of germ cells. The composition of testicular fluid may differ considerably from that of blood plasma and it is valid as well for steroids [18,19].

In this paper we attempted to determine and compare the concentrations major estrogens together with BPA in both body fluids. Our results of E2 in blood plasma are in accordance with those of other authors in normospermic men [20–22]. In this case, the results from immunoassays are comparable with instrumental methods. Few studies have determined E2 in seminal fluid in normospermic men [20,21,23–25] (for review see [18,19]). Seminal E2 concentrations were substantially higher than concentrations in blood plasma when using competitive immunoassays [20,21,23,26]. It was concluded that immunoassays are suitable for blood plasma determinations of E2, but are not applicable for seminal plasma E2 determination without more selective chromatographic separation [24].

When comparing our results to chromatographic determinations, the results are more similar [24,25]. None of these authors, however, measured simultaneously plasma levels of E2, therefore it was not possible to correlate concentrations of E2 in both biological fluids.

E1 is a biologically less active estrogen with the concentration range in plasma/serum of 9–60 pg/mL [22,27–29]. The role of E1 in men is unclear. Of interest may be the recent finding of Jasuja et al. with a cohort of 1458 men that plasma E1 was associated with diabetes risk [29]. Here we reported a median of 25 pg/mL in blood plasma. To our knowledge, only three studies measured E1 in seminal fluid. The reported mean concentration in normospermic men amounted as much as 150–178 pg/mL [24,26] in comparison to only 5 pg/mL found by us. Our data are almost 30 times less than results published in the 80s probably due to the instrumentation at the time.

E3 is the third, weakest bioactive estrogen. Mean concentrations in blood plasma in healthy men were reported between 19.1 [30] and 36.8 pg/mL [31], which is in accordance with our findings. To our knowledge, until now, nobody has measured E3 in the seminal fluid. The nearly twice higher concentration of E3 in seminal fluid than in blood plasma (74 pg/mL in seminal fluid vs 38.4 pg/mL in plasma) could be a result either of more intensive metabolic degradation of E1 in the testes or in seminal fluid, or of easier transport of E3 to seminal fluid.

Many studies reported serum/plasma levels of BPA in men ([32–37]; for review see [2,38]). The determination of BPA in body fluids is more problematic in comparison with measurements of steroids due to the possible BPA contamination of samples during collection, handling and storage. It is necessary to check if BPA is leaking from the laboratory equipment. In our study, we evaluated potential contamination of the sample during the entire process and concluded that the total BPA contamination is below the limit of detection. Therefore, our method provides sensitive and accurate measurements.

Previously, three methods determined BPA in semen with inconsistent results [33,39,40]. Inoue et al. developed the instrumental method to quantify BPA in human semen without a derivatization step, that is why the LOD was relatively high – 100 pg/mL and LOQ 500 pg/mL [39]. The authors compared their method with the ELISA method and concluded that the ELISA results may give inaccurate

Table 5
Concentration ranges of each analyte and medians of measured samples ($n=79$) in both fluids.

Analyte	Plasma		Seminal fluid	
	Concentration range (ng/ml)	Median (ng/ml)	Concentration range (ng/ml)	Median (ng/ml)
BPA	< LLOQ–7.23	0.093	< LLOQ–10.9	0.085
E2	< LLOQ–0.073	0.022	< LLOQ–0.258	0.005
E1	< LLOQ–0.065	0.025	< LLOQ–0.063	0.006
E3	< LLOQ–0.298	0.018	< LLOQ–0.360	0.043

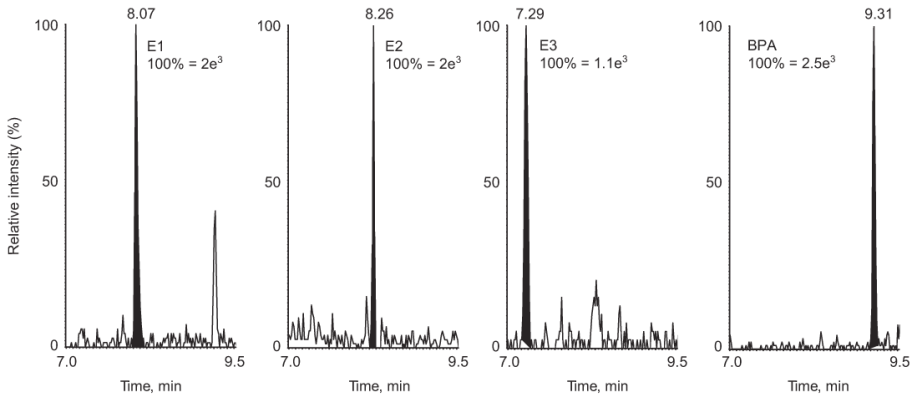


Fig. 2. Chromatograms of estrogens in plasma and BPA in seminal fluid. The measured concentrations were as follows: E1 – 0.148 ng/ml; E2 – 0.056 ng/ml; E3 – 0.149 ng/ml; BPA – 0.0723 ng/ml.

results due to the matrix effect and insufficient specificity of anti-BPA antibody [33,39,41]. Other authors also measured BPA in seminal plasma, but all of their samples were surprisingly below LOD (1 pg/mL) [40]. The third study concerned BPA levels in follicular and seminal fluids by RIA in 28 randomly selected couples attending in vitro fertilization. The concentration range of BPA in semen varied from 80 pg/mL to 1 ng/mL [33].

Our method with LLOQ 28.9 pg/mL enabled us to measure unconjugated BPA in seminal fluid in 87% of samples. Median of BPA concentration in seminal fluid is similar with the concentrations of BPA in human plasma (see Table 5), which indicates that the transmission from blood through blood–testis barrier to seminal plasma occurs. Nevertheless, plasma and seminal fluid concentrations of BPA did not correlate with each other, which emphasized that seminal plasma and testis represent quite a different milieu. It points to importance of simultaneous determination of BPA as an ED and steroids in semen. Furthermore, a trend of increasing seminal E2 and E1 levels as well as plasma E2 levels towards the highest BPA quartile was observed (data not shown).

5. Conclusion

To the best of our knowledge, this is the first method that allows estrogens and BPA to be measured together in one run. The concentrations of E1, E2 and for the first time also of E3 in seminal plasma are reported. The method will be beneficial for the monitoring of estrogen metabolism within the context of BPA exposure in men and their fertility status. The method enables highly sensitive BPA and estrogens to be determined and the examination disruption of estrogen metabolic pathways by BPA not only in the circulation but also directly in testes.

Acknowledgements

This work was supported by Internal Grant Agency Project no. NT/13369 from the Czech Ministry of Health.

References

[1] F.S. Vom Saal, W.V. Welshons, Mol. Cell. Endocrinol. 398 (1–2) (2014) 101–113.
[2] L.N. Vandenberg, R. Hauser, M. Marcus, N. Olea, W.V. Welshons, Reprod. Toxicol. 24 (2) (2007) 139–177.
[3] B.S. Rubin, Bisphenol A, J. Steroid. Biochem. Mol. Biol. 127 (1-2) (2011) 27–34.
[4] J.D. Meeker, S. Ehrlich, T.L. Toth, D.L. Wright, A.M. Calafat, A.T. Trisini, X. Ye, R. Hauser, Reprod. Toxicol. 30 (4) (2010) 532–539.
[5] D.K. Li, Z. Zhou, M. Miao, Y. He, J. Wang, J. Ferber, L.J. Herrinton, E. Gao, W. Yuan, Fertil. Steril. 95 (2) (2011) 625–630 e1–4.
[6] T.H. Lassen, H. Frederiksen, T.K. Jensen, J.H. Petersen, U.N. Joensen, K.M. Main, N.E. Skakkebaek, A. Juul, N. Jorgensen, A.M. Andersson, Environ. Health Perspect. 122 (5) (2014) 478–484.
[7] J. Peretz, L. Vrooman, W.A. Ricke, P.A. Hunt, S. Ehrlich, R. Hauser, V. Padmanabhan, H.S. Taylor, S.H. Swan, C.A. VandeVoort, J.A. Flaws, Environ. Health Perspect. 122 (8) (2014) 775–786.
[8] T. Galloway, R. Cipelli, J. Guralnik, L. Ferrucci, S. Bandinelli, A.M. Corsi, C. Money, P. McCormack, D. Melzer, Environ. Health Perspect. 118 (11) (2010) 1603–1608.
[9] J.D. Meeker, A.M. Calafat, R. Hauser, Environ. Sci. Technol. 44 (4) (2010) 1458–1463.
[10] J. Mendiola, N. Jorgensen, A.M. Andersson, A.M. Calafat, X. Ye, J.B. Redmon, E.Z. Drobnis, C. Wang, A. Sparks, S.W. Thurston, F. Liu, S.H. Swan, Environ. Health Perspect. 118 (9) (2010) 1286–1291.
[11] C.A. Richter, J.A. Taylor, R.L. Ruhlen, W.V. Welshons, F.S. Vom Saal, Environ. Health Perspect. 115 (6) (2007) 902–908.
[12] H.J. Lee, S. Chattopadhyay, E.Y. Gong, R.S. Ahn, K. Lee, Toxicol. Sci. 75 (1) (2003) 40–46.
[13] M.R. Anari, R. Bakhtiar, B. Zhu, S. Huskey, R.B. Franklin, D.C. Evans, Anal. Chem. 74 (16) (2002) 4136–4144.
[14] Guidance for Industry, Bioanalytical method Validation. Available from: ⟨http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070107.pdf⟩, 2001 (accessed 03.02.15).
[15] T. Higashi, A. Goto, M. Morohashi, S. Ogawa, K. Komatsu, T. Sugiura, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 969 (2014) 230–234.
[16] M. Hill, A. Parizek, R. Kancheva, M. Duskova, M. Velikova, L. Kriz, M. Klimkova, A. Paskova, Z. Zizka, P. Matucha, M. Meloun, L. Starka, J. Steroid Biochem. Mol. Biol. 121 (3–5) (2010) 594–610.
[17] R.S. Wang, S. Yeh, C.R. Tzeng, C. Chang, Endocr. Rev. 30 (2) (2009) 119–132.
[18] R. Hampl, J. Kubatova, J. Heracek, V. Sobotka, L. Starka, Endocr. Regul. 47 (3) (2013) 149–158.
[19] R. Hampl, J. Kubatova, V. Sobotka, J. Heracek, Horm. Mol. Biol. Clin. Investig. 13 (1) (2013) 1–5.
[20] L. Bujan, R. Mieusset, F. Audran, S. Lumbroso, C. Sultan, Hum. Reprod. 8 (1) (1993) 74–77.
[21] R. Luboshitzky, Z. Shen-Orr, P. Herer, Arch. Androl. 48 (3) (2002) 225–232.
[22] F. Giton, S. Trabado, L. Maione, J. Sarfati, Y. Le Bouc, S. Brailly-Tabard, J. Fiet, J. Young, J. Clin. Endocrinol. Metab. (2014) jc20142658.
[23] Q. Zhang, Q. Bai, Y. Yuan, P. Liu, J. Qiao, J. Androl. 31 (2) (2010) 215–220.
[24] A. Reiffsteck, L. Dehennin, R. Scholler, J. Steroid Biochem. 17 (5) (1982) 567–572.
[25] A. Zalata, M. El-Mogy, A. Abdel-Khabir, Y. El-Bayoumy, M. El-Baz, T. Mostafa, Andrologia 46 (7) (2014) 761–765.
[26] D. Adamopoulos, D.M. Lawrence, P. Vassilopoulos, N. Kapolla, L. Kontogeorgos, H.H. McGarrigle, J. Clin. Endocrinol. Metab. 59 (3) (1984) 447–452.
[27] Mayo Clinic Mayo Medical Laboratories. Available from: ⟨http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/84230⟩, 2015 (accessed 03.02.15).
[28] M.M. Kushnir, A.L. Rockwood, J. Bergquist, M. Varshavsky, W.L. Roberts, B. Yue, A.M. Bunker, A.W. Meikle, Am. J. Clin. Pathol. 129 (4) (2008) 530–539.
[29] G.K. Jasuja, T.G. Travison, M. Davda, A.J. Rose, A. Zhang, M.M. Kushnir, A. L. Rockwood, W. Meikle, A.D. Coviello, R. D'Agostino, R.S. Vasan, S. Bhasin, Diabetes Care 36 (9) (2013) 2591–2596.
[30] O.L. Myking, O. Digranes, J. Steroid Biochem. 20 (3) (1984) 799–801.
[31] J.R. Green, N.A. Mowat, R.A. Fisher, D.C. Anderson, Gut 17 (6) (1976) 426–430.
[32] M.P. Zhao, Y.Z. Li, Z.Q. Guo, X.X. Zhang, W.B. Chang, Talanta 57 (6) (2002) 1205–1210.
[33] N. Kaddar, N. Bendridi, C. Harthe, M.R. de Ravel, A.L. Bienvenu, C.Y. Cuilleron, E. Mappus, M. Pugeat, H. Dechaud, Anal. Chim. Acta 645 (1–2) (2009) 1–4.
[34] M.S. Bloom, F.S. Vom Saal, D. Kim, J.A. Taylor, J.D. Lamb, V.Y. Fujimoto, Environ. Toxicol. Pharmacol. 32 (2) (2011) 319–323.
[35] W. Aekplakorn, L.O. Chailurkit, B. Ongphiphadhanakul, J. Diabetes 7 (2) (2015) 240–249.
[36] H.T. Wan, P.Y. Leung, Y.G. Zhao, X. Wei, M.H. Wong, C.K. Wong, J. Hazard. Mater. 261 (2013) 763–769.
[37] J. Sajiki, K. Takahashi, J. Yonekubo, J. Chromatogr. B Biomed. Sci. Appl. 736 (1–2) (1999) 255–261.
[38] L.N. Vandenberg, I. Chahoud, J.J. Heindel, V. Padmanabhan, F.J. Paumgartten, G. Schoenfelder, Environ. Health Perspect. 118 (8) (2010) 1055–1070.
[39] K. Inoue, M. Wada, T. Higuchi, S. Oshio, T. Umeda, Y. Yoshimura, H. Nakazawa, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 773 (2) (2002) 97–102.
[40] M. Katayama, Y. Matsuda, K.I. Shimokawa, H. Ishikawa, S. Kaneko, Anal. Lett. 36 (12) (2003) 2659–2667.
[41] H. Fukata, H. Miyagawa, N. Yamazaki, C. Mori, Toxicol. Mech. Methods 16 (8) (2006) 427–430.

PŘÍLOHA IX

Vitku J, Sosvorova L, Chlupacova T, Hampl R, Hill M, Sobotka V, Heracek J, Bicikova M, Starka L: Differences in bisphenol A and estrogen levels in the plasma and seminal plasma of men with different degrees of infertility, Phys Res, 64 (Suppl. 2) S303 - S311 (2015). IF = 1.293

Physiol. Res. 64 (Suppl. 2): S303-S311, 2015

Differences in Bisphenol A and Estrogen Levels in the Plasma and Seminal Plasma of Men With Different Degrees of Infertility

J. VITKU[1], L. SOSVOROVA[1], T. CHLUPACOVA[1], R. HAMPL[1], M. HILL[1], V. SOBOTKA[2], J. HERACEK[3,4], M. BICIKOVA[1], L. STARKA[1]

[1]Department of Steroids and Proteofactors, Institute of Endocrinology, Prague, Czech Republic, [2]Department of Urology, Third Faculty of Medicine, Charles University in Prague, Czech Republic, [3]Department of Urology, First Faculty of Medicine, Charles University in Prague, Czech Republic, [4]Department of Urology, Military University Hospital Prague, Czech Republic

Received June 2, 2015
Accepted June 16, 2015

Summary

The general population is potentially exposed to many chemicals that can affect the endocrine system. These substances are called endocrine disruptors (EDs), and among them bisphenol A (BPA) is one of the most widely used and well studied. Nonetheless, there are still no data on simultaneous measurements of various EDs along with steroids directly in the seminal fluid, where deleterious effects of EDs on spermatogenesis and steroidogenesis are assumed. We determined levels of BPA and 3 estrogens using LC-MS/MS in the plasma and seminal plasma of 174 men with different degrees of infertility. These men were divided according their spermiogram values into 4 groups: (1) healthy men, and (2) slightly, (3) moderate, and (4) severely infertile men. Estradiol levels differed across the groups and body fluids. Slightly infertile men have significantly higher BPA plasma and seminal plasma levels in comparison with healthy men ($p<0.05$ and $p<0.01$, respectively). Furthermore, seminal BPA, but not plasma BPA, was negatively associated with sperm concentration and total sperm count (–0.27; $p<0.001$ and –0.24; $p<0.01$, respectively). These findings point to the importance of seminal plasma in BPA research. Overall, a disruption of estrogen metabolism was observed together with a weak but significant impact of BPA on sperm count and concentration.

Key words

Bisphenol A • Estrone • Estradiol • Estriol • Seminal fluid/plasma • Blood plasma • Infertile men • LC-MS

Corresponding author

J. Vitku, Department of Steroids and Proteofactors, Institute of Endocrinology, Národní 8, 116 94, Prague 1, Czech Republic. E-mail: jvitku@endo.cz

Introduction

Bisphenol A (BPA) is a long- and well-known endocrine disruptor (ED), that still receives a considerable amount of attention from the scientific community as well as the general public, mainly because of its ubiquity in our environment and uncertainties about its effects on humans. For the most part, BPA enters the body by the ingestion of contaminated food or beverages. It leaks from polycarbonate plastics, which are used to line food and drink containers such as bottles and cans. Further minor ways of penetrating into the body are through the skin (e.g. contact with thermal receipts) (Ehrlich *et al.* 2014, Liao and Kannan 2011) or inhalation (e.g. cigarette smoke or dust) (Braun *et al.* 2011, He *et al.* 2009, Inoue *et al.* 2006, Rudel *et al.* 2003). There is still an ongoing debate whether environmental levels of BPA are harmful for the population or not.

BPA is a weak estrogen when considering its binding activities to the estrogen receptor (ER) (Welshons *et al.* 2003). On the other hand, it can act with the same potency as endogenous estradiol (E2) on the non-classical membrane estrogen receptor (Alonso-Magdalena *et al.* 2012, Quesada *et al.* 2002, Wozniak *et al.* 2005). Its mode of action, however, is much more complex. It may act through other nuclear receptors

including the estrogen related receptor (Delfosse *et al.* 2014, Okada *et al.* 2008), androgen receptor (Lee *et al.* 2003, Teng *et al.* 2013), thyroid receptor (Moriyama *et al.* 2002), glucocorticoid receptor (Sargis *et al.* 2010), peroxisome proliferator activated receptor γ (PPARγ) (Pereira-Fernandes *et al.* 2013, Wang *et al.* 2010) and pregnane X receptor (Sui *et al.* 2012). An interaction of BPA with the expression and activity of steroidogenic enzymes has also been reported (Cannon *et al.* 2000, Gilibili *et al.* 2014, Hanioka *et al.* 1998, Ye *et al.* 2014, Ye *et al.* 2011, Zhang *et al.* 2011). Moreover, BPA exerts a non-monotonic dose response at low physiologically-relevant concentrations, with tissue-specific effects (for review see Wetherill *et al.* 2007).

Endogenous estrogens are thought to have an important role in the testis, because estrogen biosynthesis occurs in the testicular cells and the absence of ERs causes adverse effects on spermatogenesis as well as steroidogenesis (Akingbemi 2005). Physiological levels of E2 are essential for normal spermatogenesis; in contrast, a surplus of estrogens (together with a lack of testosterone) occurs in infertility (Pavlovich *et al.* 2001). The impact of BPA on male reproductive function is of particular interest due to its estrogenic and antiandrogenic activities, which could have potentially deleterious effects on spermatogenesis (reviewed in Hampl *et al.* 2013a,b).

Recently, our group developed a sensitive and accurate method for the simultaneous measurement of estrogens (estrone, estradiol and estriol) and BPA in human plasma and seminal fluid (Vitku *et al.* 2015). Reported levels of estrogens and BPA vary in these biological fluids in normospermic men, underlining the fact that seminal fluid is a unique environment where the effects of BPA may be expressed directly in the testis. In this study, we aimed to investigate BPA and estrogen concentrations in the plasma and seminal fluid in men with different degrees of infertility, and evaluate the potential effects of BPA on the estrogen metabolism and sperm quality.

Materials and Methods

Chemicals and reagents

Reference standards of estrone (E1), 17β-estradiol (E2) and estriol (E3) and deuterated standards of estrone (d4E1) and estriol (d2E3) were purchased from Steraloids (Newport, RI, USA). Bisphenol A (BPA), deuterated BPA (d16BPA) and deuterated E2 (d3E2) were obtained from Sigma-Aldrich (St. Louis, MO, USA) as were 99.9 % tert-butyl methyl ether (MTBE), acetone, sodium bicarbonate, sodium hydroxide and dansyl chloride. Methanol and water for chromatography were purchased from Merck (Darmstadt, Germany). All solvents and reagents were of HPLC grade.

Study population and sample collection

Samples of plasma and seminal plasma were obtained from patients attending the Centre of Assisted Reproduction Pronatal (Prague, CZ). Each patient underwent a standardized ejaculate examination (spermiogram) according to the World Health Organization (WHO) 2010 criteria. In a previous study (Vitku *et al.* 2015), we dealt with the problem of ensuring that sampling equipment is not contaminated with BPA. Following the procedures detailed in that study, all steps in sample collection and processing were carried out using BPA-free glass equipment and stored in glass tubes in –20 °C until analysis. Plasma and seminal plasma samples were obtained from 174 men with different degrees of infertility. The mean age of the men was 35.97±5.64 years and BMI 27.32±3.65. Men were divided into four groups according to spermiogram values. The first group included normospermic men with a normal spermiogram (n=84); oligospermic, asthenospermic and oligoasthenospermic men were included in the second group (n=56); teratospermic, oligoasthenoteratospermic and oligoteratospermic men were placed in the third group (n=20); and the fourth group consisted of azoospermic men (n=14). We termed these groups: (1) healthy men, and (2) slightly, (3) moderately and (4) severely infertile men.

The study was performed in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The protocol was approved by the Ethical Committee of the Institute of Endocrinology. Informed and written consent with the use of biological materials for research reasons was obtained from all subjects participating in the project.

Determination of estrogens and BPA

We analyzed unconjugated forms of E1, E2, E3 and BPA in plasma and seminal plasma by a newly developed isotope dilution ultra high performance liquid chromatography – mass spectrometry method (Vitku *et al.* 2015). A Kinetex C18 column (100 x 3.0 mm, 1.7 µm; Phenomenex, Torrance, CA, USA) and Security Guard ULTRA cartridge system (UHPLC C18 for 3 mm ID column; Phenomenex, Torrance, CA, USA) was used for

the analysis. An Eksigent ultraLC 110 liquid chromatograph system (Redwood City, CA, USA) was coupled to an API 3200 mass spectrometer (AB Sciex, Concord, Canada) with an electrospray ionization (ESI) probe operating in positive mode.

Detailed information about the analysis procedure and validation are provided elsewhere (Vitku *et al.* 2015). Briefly, 500 µl of plasma or 1000 µl of seminal fluid was diluted by 500 µl of physiological solution (0.9 % sodium chloride) and samples were vortexed. Consequently, extraction with 2 ml of 99.9 % tert-butyl methyl ether (MTBE) for one minute was performed. The organic phase was evaporated until dryness using a vacuum concentrator (55 °C). Further, a derivatization step was carried out: a volume of 50 µl of sodium bicarbonate buffer (100 mM, pH 10.5) and 50 µl of dansyl chloride in acetone (1 mg/ml) were added to the dry residues, shortly vortexed and incubated in a heat block (60 °C) for 5 min. After removing from the heat block, samples were left to cool down to room temperature and again evaporated until dryness. Thereafter, samples were reconstituted with 300 µl of methanol, and 50 µl were transferred to the glass insert containing 50 µl of the ammonium formate in ultrapure water (10 mM) pre-pipetted. The injection volume was 50 µl.

Statistical evaluation

Before statistical analysis, the data were transformed by Box-Cox transformation due to the significant skewness, kurtosis and heteroscedasticity of most variables. Differences between groups were evaluated using one-way ANOVA followed by least square difference multiple comparisons. The statistical software Statgraphics Centurion XVI from Statpoint Inc. (Warrenton, VA, USA) was used for data transformations, correlations, ANOVA testing and multiple comparisons. Multiple outliers for correlations were found using NCSS 2007 (Kaysville, UT, USA).

Results

Here we report the first data on BPA exposure in a population of Czech men (Table 1). The groups of men did not significantly differ from each other in age and BMI. We detected BPA, E2, E1 and E3 in 87 %, 94 %, 100 % and 62 % of plasma samples, respectively, and 92 %, 84 %, 90 % and 97 % of seminal samples, respectively. In the rest of the samples, levels were under the lower limit of quantification.

Table 1. Comparisons of age, BMI, BPA (pg/ml) and estrogen levels (pg/ml) in groups of men with different degrees of infertility.

		Group 1 (n=84)	Group 2 (n=56)	Group 3 (n=20)	Group 4 (n=14)	p-value	Multiple comparisons
	Age	36 (34.9; 36.7)	35.8 (34.5; 37.2)	35.7 (33.4; 37.9)	35.2 (32.5; 37.9)	0.959	
	BMI	27.4 (26.2;28.5)	27.5 (26.2;28.7)	26.6 (24.9; 28.2)	26.2 (24.9; 28.2)	0.451	
Plasma	BPA	47 (26;81)	137 (75; 239)	114 (42; 270)	33 (6;125)	**0.036**	1<2
	E2	22 (18;26)	18 (15;23)	17 (11;24)	7 (3;12)	**0.002**	1,2,3>4
	E1	24 (20;29)	23 (18;28)	21 (15;29)	17 (10;26)	0.513	
	E3	19 (11;31)	20 (10;36)	19 (5;53)	13 (3;36)	0.933	
Seminal fluid	BPA	66 (44;94)	144 (98;205)	132 (69;228)	179 (84;330)	**0.009**	1<2,4
	E2	2 (1;3)	4 (3;6)	9 (5;15)	7 (3;14)	**0.002**	1<3,4; 2<3
	E1	4 (3;5)	5 (4;7)	9 (6;13)	6 (3;9)	**0.008**	1,2<3
	E3	43 (30;59)	31 (19;47)	34 (16;63)	83 (40;154)	0.129	2<4

Data are shown as means and 95.0 percent confidence intervals (in parentheses) for each group, with levels of significance for the ANOVA and multiple comparisons provided. Group 1 = normospermic men; Group 2 = oligospermic/asthenospermic/ oligoasthenospermic men; Group 3 = teratospermic/oligoteratospermic/oligoasthenoteratospermic men; Group 4 = azoospermic men.

Comparisons of plasma and seminal BPA and estrogen levels in men with different degrees of infertility

Generally, BPA levels in seminal fluid were slightly higher than those in plasma, except for in the 4th group where seminal fluid BPA levels were nearly three times greater (Table 1). Seminal BPA concentrations were found to be higher in all groups of men with various degrees of infertility in comparison with normospermic

men. Plasma BPA levels were significantly higher in the group of slightly infertile men compared to healthy men. Concentrations of plasma E2 decreased from the first to the fourth group, while seminal E2 levels increased. Mean plasma levels of E2 varied from 7 to 22 pg/ml among the groups, in comparison with 2-7 pg/ml mean E2 in semen. Plasma and seminal fluid E1 levels showed similar results as for E2. Concentrations of E3 in seminal fluid were significantly higher than E3 plasma levels in all groups, and did not differ across the groups. A graphical representation of the differences in BPA and E2 levels in both fluids across the groups is shown in Figure 1.

Correlation matrix between BPA, estrogens and spermiogram parameters

Pearson´s correlation coefficients between all parameters are provided in Table 2. Although plasma and seminal BPA levels correlate with each other, only BPA in seminal fluid was negatively associated with sperm concentration and total sperm count. This indicates the importance of seminal fluid in research on the effects of BPA. Another finding that is of interest is the positive association between BPA and E2 in both body fluids.

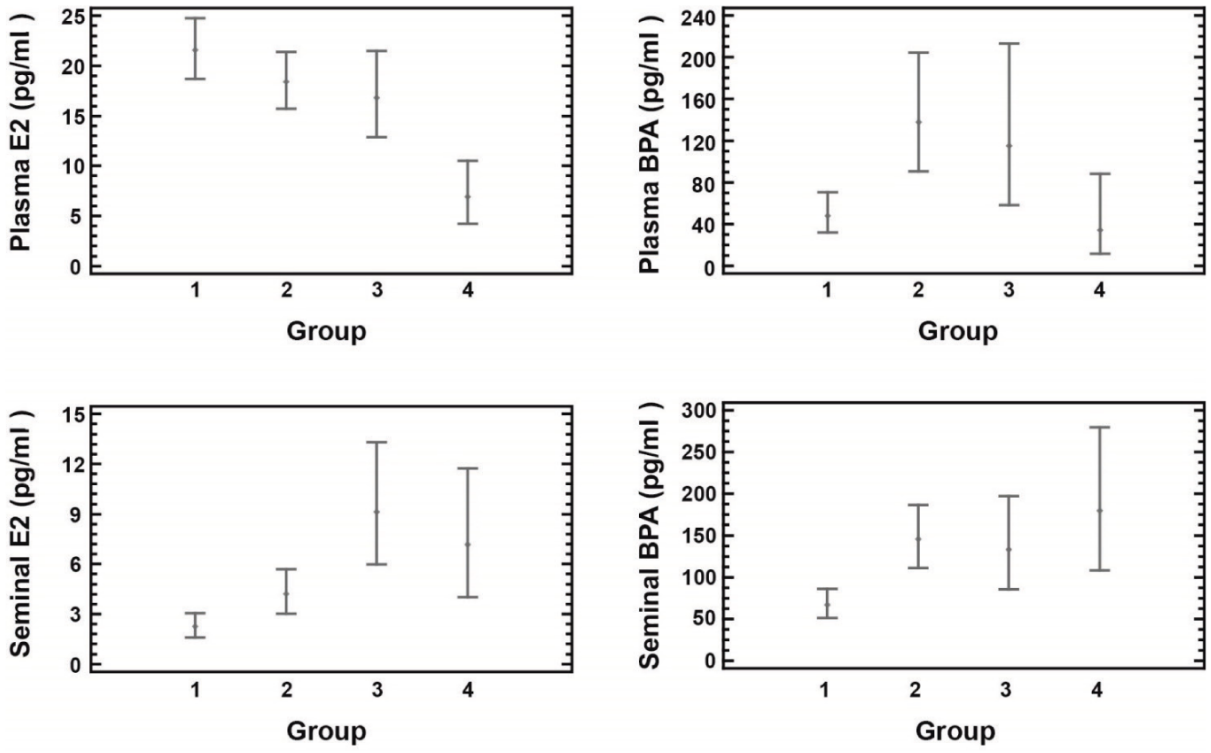


Fig. 1. Differences in bisphenol A (BPA) and estradiol (E2) levels in plasma and seminal plasma among the 4 groups of men. Group 1 = normospermic men (n=84); Group 2 = oligospermic/asthenospermic/oligoasthenospermic men (n=56); Group 3 = teratospermic/ oligoteratospermic/oligoasthenoteratospermic men (n=20); Group 4= azoospermic men (n=14).

Discussion

To the best of our knowledge, this is the first study reporting levels of EDs and steroids directly in seminal fluid. We measured unconjugated forms of estrogens as well as BPA, thought to be the active forms, and found that the levels differ in seminal fluid and plasma. This indicates that seminal fluid is a unique milieu that deserves further investigation.

Experimental studies in adult rodents have provided evidence that exposure to BPA affects sperm quality and production (reviewed in Peretz *et al.* 2014). However, few studies have reported the impact of BPA on sperm quality in adult men. To our knowledge, six studies have dealt with this problem, all measuring BPA in urine, and with divergent results (Goldstone *et al.* 2014, Knez *et al.* 2014, Lassen *et al.* 2014, Li *et al.* 2011, Meeker *et al.* 2010b, Mendiola *et al.* 2010). Our finding of decreasing sperm concentrations and counts in association with increasing seminal BPA (Table 2) is in

agreement with three of the studies (Knez *et al.* 2014, Li *et al.* 2011, Meeker *et al.* 2010b). In contrast, the other studies did not find any association between these sperm parameters and urinary BPA (Goldstone *et al.* 2014, Mendiola *et al.* 2010). However, Lassen *et al.* (2014) reported a significant inverse association between BPA excretion and progressive motility. Still, the outcomes vary across studies, apparently due to the difficulties in evaluating the effects of EDs when organisms are exposed more than one at a time.

Table 2. Pearson´s correlation coefficients among levels of plasma and seminal BPA and estrogens, and spermiogram parameters. Correlation coefficients are provided in the first row of each variable, p-values are in the second row. If significant, coefficients and p-values are highlighted in bold.

	Plasma E2	**Plasma E1**	**Plasma E3**	**Plasma BPA**	**Seminal E2**	**Seminal E1**	**Seminal E3**	**Seminal BPA**	**Concentration**	**Total count**	**Motility**	**Progressive motility**	**Morphology**
Plasma E2	1.000												
Plasma E1	**0.372** **0.000**	1.000											
Plasma E3	**0.366** **0.001**	0.173 0.144	1.000										
Plasma BPA	**0.363** **0.000**	-0.146 0.120	0.192 0.103	1.000									
Seminal E2	-0.065 0.498	**-0.236** **0.012**	0.190 0.110	0.076 0.378	1.000								
Seminal E1	0.035 0.714	-0.004 0.967	0.124 0.299	0.058 0.499	**0.181** **0.023**	1.000							
Seminal E3	**0.229** **0.016**	-0.059 0.538	**0.384** **0.001**	0.106 0.218	**0.192** **0.016**	**0.158** **0.047**	1.000						
Seminal BPA	0.082 0.395	**-0.257** **0.006**	0.185 0.120	**0.338** **0.000**	**0.318** **0.000**	0.061 0.450	0.156 0.051	1.000					
Concentration	-0.006 0.952	0.053 0.575	-0.003 0.983	-0.160 0.057	-0.146 0.066	-0.098 0.222	0.063 0.429	**-0.271** **0.001**	1.000				
Total count	-0.059 0.536	0.077 0.412	-0.022 0.852	-0.163 0.052	**-0.164** **0.039**	-0.106 0.184	0.010 0.898	**-0.236** **0.003**	**0.957** **0.000**	1.000			
Motility	**0.293** **0.002**	0.164 0.081	**0.268** **0.022**	0.065 0.439	**-0.248** **0.002**	**-0.187** **0.019**	0.021 0.796	-0.115 0.151	**0.513** **0.000**	**0.506** **0.000**	1.000		
Progressive motility	**0.291** **0.002**	0.172 0.066	**0.231** **0.049**	0.012 0.886	**-0.244** **0.002**	**-0.225** **0.005**	0.027 0.732	-0.129 0.106	**0.546** **0.000**	**0.550** **0.000**	**0.958** **0.000**	1.000	
Morphology	0.096 0.310	-0.010 0.918	0.133 0.264	-0.103 0.223	**-0.203** **0.010**	**-0.185** **0.020**	0.013 0.870	-0.124 0.119	**0.595** **0.000**	**0.561** **0.000**	**0.722** **0.000**	**0.741** **0.000**	1.000

The levels of BPA in the plasma of group 4 are similar to those of group1, i.e. of normal fertile men. One possible reason may be that the severe infertility in men of group 4 is caused by other factors than the effects of hormones and other constituents of seminal fluid, e.g. genetic or anatomic causes or the result of infections.

Although the body of ED research is continuously expanding, there still exist uncertainties in the process of BPA degradation in the body. It has generally been thought that BPA is rapidly metabolized in the liver and excreted in the urine within hours (Volkel *et al.* 2002, 2005). On the other hand, a recent study

showed that during fasting BPA levels did not decline rapidly, suggesting a substantial non-food-related exposure or accumulation in tissues (Stahlhut *et al.* 2009). This is why we decided to study the relationships between EDs and sperm quality in seminal fluid, with its closer proximity to sperm production. According to our previous study, seminal and plasma BPA levels in normospermic men did not correlate with each other, indicating that their distribution or metabolism are different in these body fluids (Vitku *et al.* 2015). Furthermore, BPA competes with sex steroids for human plasma SHBG (Dechaud *et al.* 1999), suggesting that the bioavailability and half-time in blood could be affected. Further studies on the persistence of BPA in semen and other body fluids during chronic exposure are needed.

Studies that have investigated associations between BPA and steroidogenesis are divergent as well. Only a few of them have investigated the impact of BPA on estrogen levels and estrogen metabolism. Urinary BPA was reported to be positively associated with plasma E2 in a group of young men (Lassen *et al.* 2014). Another study showed no association with E2 levels (Galloway *et al.* 2010) and another reported an inverse relationship (Meeker *et al.* 2010a).

Our results show that the levels of E2 differ across the groups and body fluids. Only 10-25 % of the E2 in circulation in men is synthesized in the testis. Aromatase activity and estrogen biosynthesis in men occur mainly in adipose tissue (Levine *et al.* 1997). In our study, the increases of E2 and E1 in the seminal plasma of increasingly infertile men and the opposite trends in plasma raise some considerations. One explanation is that peripheral estrogens penetrate more easily through the blood-testis-barrier to the testis. Alternatively, estrogens originating in the testis may have a harder time accessing the periphery. Other possibilities include the increased expression or activity of aromatase in the testis and/or decreased expression or activity in the adipose tissue. The metabolism of E2 could be also protracted by the interaction of BPA with enzymes involved in steroid conjugation such as estrogen glucuronidase or sulfotransferase. This explanation is in accordance with the *in vitro* study of Zhang *et al.* (2011), who reported that BPA suppressed E2 catabolism in H295R cells but without altering aromatase activity. On the other hand, studies from *in vitro* and *in vivo* experiments focused on the impact of BPA on aromatase activity have yielded conflicting results (Castro *et al.* 2013, Ehrlich *et al.* 2013, Rajakumar *et al.* 2015, Shanthanagouda *et al.* 2014).

In conclusion, the results of our study show an inverse association between seminal BPA and sperm count and concentration. The correlation coefficients were relatively weak ($r=-0.24$ and $r=-0.27$, respectively) suggesting that BPA slightly, but significantly, contributes to the final state of sperm quality, together with other factors. Moreover, a disruption of estrogen metabolism was observed, but the mechanism of action remains to be elucidated.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This study was supported by Internal Grant Agency Project no. NT/13369 from the Czech Ministry of Health.

References

AKINGBEMI BT: Estrogen regulation of testicular function. *Reprod Biol Endocrinol* **3**: 51, 2005.

ALONSO-MAGDALENA P, ROPERO AB, SORIANO S, GARCIA-AREVALO M, RIPOLL C, FUENTES E, QUESADA I, NADAL A: Bisphenol-A acts as a potent estrogen via non-classical estrogen triggered pathways. *Mol Cell Endocrinol* **355**: 201-207, 2012.

BRAUN JM, KALKBRENNER AE, CALAFAT AM, BERNERT JT, YE X, SILVA MJ, BARR DB, SATHYANARAYANA S, LANPHEAR BP: Variability and predictors of urinary bisphenol A concentrations during pregnancy. *Environ Health Perspect* **119**: 131-137, 2011.

CANNON JM, KOSTORYZ E, RUSSO KA, SMITH RE, YOURTEE DM: Bisphenol A and its biomaterial monomer derivatives alteration of in vitro cytochrome P450 metabolism in rat, minipig, and human. *Biomacromolecules* **1**: 656-664, 2000.

CASTRO B, SANCHEZ P, TORRES JM, PREDA O, DEL MORAL RG, ORTEGA E: Bisphenol A exposure during adulthood alters expression of aromatase and 5alpha-reductase isozymes in rat prostate. *PLoS One* **8**: e55905, 2013.

DECHAUD H, RAVARD C, CLAUSTRAT F, DE LA PERRIERE AB, PUGEAT M: Xenoestrogen interaction with human sex hormone-binding globulin (hSHBG). *Steroids* **64**: 328-334, 1999.

DELFOSSE V, GRIMALDI M, LE MAIRE A, BOURGUET W, BALAGUER P: Nuclear receptor profiling of bisphenol-a and its halogenated analogues. *Vitam Horm* **94**: 229-251, 2014.

EHRLICH S, WILLIAMS PL, HAUSER R, MISSMER SA, PERETZ J, CALAFAT AM, FLAWS JA: Urinary bisphenol A concentrations and cytochrome P450 19 A1 (Cyp19) gene expression in ovarian granulosa cells: an in vivo human study. *Reprod Toxicol* **42**: 18-23, 2013.

EHRLICH S, CALAFAT AM, HUMBLET O, SMITH T, HAUSER R: Handling of thermal receipts as a source of exposure to bisphenol A. *JAMA* **311**: 859-860, 2014.

GALLOWAY T, CIPELLI R, GURALNIK J, FERRUCCI L, BANDINELLI S, CORSI AM, MONEY C, MCCORMACK P, MELZER D: Daily bisphenol A excretion and associations with sex hormone concentrations: results from the InCHIANTI adult population study. *Environ Health Perspect* **118**: 1603-1608, 2010.

GILIBILI RR, VOGL AW, CHANG TK, BANDIERA SM: Localization of cytochrome P450 and related enzymes in adult rat testis and downregulation by estradiol and bisphenol A. *Toxicol Sci* **140**: 26-39, 2014.

GOLDSTONE AE, CHEN Z, PERRY MJ, KANNAN K, LOUIS GM: Urinary bisphenol A and semen quality, the LIFE Study. *Reprod Toxicol* **51C**: 7-13, 2014.

HAMPL R, KUBATOVA J, HERACEK J, SOBOTKA V, STARKA L: Hormones and endocrine disruptors in human seminal plasma. *Endocr Regul* **47**: 149-158, 2013a.

HAMPL R, KUBATOVA J, SOBOTKA V, HERACEK J: Steroids in semen, their role in spermatogenesis, and the possible impact of endocrine disruptors. *Horm Mol Biol Clin Investig* **13**: 1-5, 2013b.

HANIOKA N, JINNO H, NISHIMURA T, ANDO M: Suppression of male-specific cytochrome P450 isoforms by bisphenol A in rat liver. *Arch Toxicol* **72**: 387-394, 1998.

HE Y, MIAO M, HERRINTON LJ, WU C, YUAN W, ZHOU Z, LI DK: Bisphenol A levels in blood and urine in a Chinese population and the personal factors affecting the levels. *Environ Res* **109**: 629-633, 2009.

INOUE K, YOSHIDA S, NAKAYAMA S, ITO R, OKANOUCHI N, NAKAZAWA H: Development of stable isotope dilution quantification liquid chromatography-mass spectrometry method for estimation of exposure levels of bisphenol A, 4-tert-octylphenol, 4-nonylphenol, tetrabromobisphenol A, and pentachlorophenol in indoor air. *Arch Environ Contam Toxicol* **51**: 503-508, 2006.

KNEZ J, KRANVOGL R, BREZNIK BP, VONCINA E, VLAISAVLJEVIC V: Are urinary bisphenol A levels in men related to semen quality and embryo development after medically assisted reproduction? *Fertil Steril* **101**: 215-221, e1-e5, 2014.

LASSEN TH, FREDERIKSEN H, JENSEN TK, PETERSEN JH, JOENSEN UN, MAIN KM, SKAKKEBAEK NE, JUUL A, JORGENSEN N, ANDERSSON AM: Urinary bisphenol A levels in young men: association with reproductive hormones and semen quality. *Environ Health Perspect* **122**: 478-484, 2014.

LEE HJ, CHATTOPADHYAY S, GONG EY, AHN RS, LEE K: Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. *Toxicol Sci* **75**: 40-46, 2003.

LEVINE AC, KIRSCHENBAUM A, GABRILOVE JL: The role of sex steroids in the pathogenesis and maintenance of benign prostatic hyperplasia. *Mt Sinai J Med* **64**: 20-25, 1997.

LI DK, ZHOU Z, MIAO M, HE Y, WANG J, FERBER J, HERRINTON LJ, GAO E, YUAN W: Urine bisphenol-A (BPA) level in relation to semen quality. *Fertil Steril* **95**: 625-630, e1-e4, 2011.

LIAO C, KANNAN K: Widespread occurrence of bisphenol A in paper and paper products: implications for human exposure. *Environ Sci Technol* **45**: 9372-9379, 2011.

MEEKER JD, CALAFAT AM, HAUSER R: Urinary bisphenol A concentrations in relation to serum thyroid and reproductive hormone levels in men from an infertility clinic. *Environ Sci Technol* **44**: 1458-1463, 2010a.

MEEKER JD, EHRLICH S, TOTH TL, WRIGHT DL, CALAFAT AM, TRISINI AT, YE X, HAUSER R: Semen quality and sperm DNA damage in relation to urinary bisphenol A among men from an infertility clinic. *Reprod Toxicol* **30**: 532-539, 2010b.

MENDIOLA J, JORGENSEN N, ANDERSSON AM, CALAFAT AM, YE X, REDMON JB, DROBNIS EZ, WANG C, SPARKS A, THURSTON SW, LIU F, SWAN SH: Are environmental levels of bisphenol a associated with reproductive function in fertile men? *Environ Health Perspect* **118**: 1286-1291, 2010.

MORIYAMA K, TAGAMI T, AKAMIZU T, USUI T, SAIJO M, KANAMOTO N, HATAYA Y, SHIMATSU A, KUZUYA H, NAKAO K: Thyroid hormone action is disrupted by bisphenol A as an antagonist. *J Clin Endocrinol Metab* **87**: 5185-5190, 2002.

OKADA H, TOKUNAGA T, LIU X, TAKAYANAGI S, MATSUSHIMA A, SHIMOHIGASHI Y: Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor-gamma. *Environ Health Perspect* **116**: 32-38, 2008.

PAVLOVICH CP, KING P, GOLDSTEIN M, SCHLEGEL PN: Evidence of a treatable endocrinopathy in infertile men. *J Urol* **165**: 837-841, 2001.

PEREIRA-FERNANDES A, DEMAEGDT H, VANDERMEIREN K, HECTORS TL, JORENS PG, BLUST R, VANPARYS C: Evaluation of a screening system for obesogenic compounds: screening of endocrine disrupting compounds and evaluation of the PPAR dependency of the effect. *PLoS One* **8**: e77481, 2013.

PERETZ J, VROOMAN L, RICKE WA, HUNT PA, EHRLICH S, HAUSER R, PADMANABHAN V, TAYLOR HS, SWAN SH, VANDEVOORT CA, FLAWS JA: Bisphenol a and reproductive health: update of experimental and human evidence, 2007-2013. *Environ Health Perspect* **122**: 775-786, 2014.

QUESADA I, FUENTES E, VISO-LEON MC, SORIA B, RIPOLL C, NADAL A: Low doses of the endocrine disruptor bisphenol-A and the native hormone 17beta-estradiol rapidly activate transcription factor CREB. *FASEB J* **16**: 1671-1673, 2002.

RAJAKUMAR C, GUAN H, LANGLOIS D, CERNEA M, YANG K: Bisphenol A disrupts gene expression in human placental trophoblast cells. *Reprod Toxicol* **53**: 39-44, 2015.

RUDEL RA, CAMANN DE, SPENGLER JD, KORN LR, BRODY JG: Phthalates, alkylphenols, pesticides, polybrominated diphenyl ethers, and other endocrine-disrupting compounds in indoor air and dust. *Environ Sci Technol* **37**: 4543-4553, 2003.

SARGIS RM, JOHNSON DN, CHOUDHURY RA, BRADY MJ: Environmental endocrine disruptors promote adipogenesis in the 3T3-L1 cell line through glucocorticoid receptor activation. *Obesity (Silver Spring)* **18**: 1283-1288, 2010.

SHANTHANAGOUDA AH, NUGEGODA D, PATIL JG: Effects of bisphenol A and fadrozole exposures on cyp19a1 expression in the Murray rainbowfish, Melanotaenia fluviatilis. *Arch Environ Contam Toxicol* **67**: 270-280, 2014.

STAHLHUT RW, WELSHONS WV, SWAN SH: Bisphenol A data in NHANES suggest longer than expected half-life, substantial nonfood exposure, or both. *Environ Health Perspect* **117**: 784-789, 2009.

SUI Y, AI N, PARK SH, RIOS-PILIER J, PERKINS JT, WELSH WJ, ZHOU C: Bisphenol A and its analogues activate human pregnane X receptor. *Environ Health Perspect* **120**: 399-405, 2012.

TENG C, GOODWIN B, SHOCKLEY K, XIA M, HUANG R, NORRIS J, MERRICK BA, JETTEN AM, AUSTIN CP, TICE RR: Bisphenol A affects androgen receptor function via multiple mechanisms. *Chem Biol Interact* **203**: 556-564, 2013.

VITKU J, CHLUPACOVA T, SOSVOROVA L, HAMPL R, HILL M, HERACEK J, BICIKOVA M, STARKA L: Development and validation of LC-MS/MS method for quantification of bisphenol A and estrogens in human plasma and seminal fluid. *Talanta* **140**: 62-67, 2015.

VOLKEL W, COLNOT T, CSANADY GA, FILSER JG, DEKANT W: Metabolism and kinetics of bisphenol a in humans at low doses following oral administration. *Chem Res Toxicol* **15**: 1281-1287, 2002.

VOLKEL W, BITTNER N, DEKANT W: Quantitation of bisphenol A and bisphenol A glucuronide in biological samples by high performance liquid chromatography-tandem mass spectrometry. *Drug Metab Dispos* **33**: 1748-1757, 2005.

WANG YF, CHAO HR, WU CH, TSENG CH, KUO YT, TSOU TC: A recombinant peroxisome proliferator response element-driven luciferase assay for evaluation of potential environmental obesogens. *Biotechnol Lett* **32**: 1789-1796, 2010.

WELSHONS WV, THAYER KA, JUDY BM, TAYLOR JA, CURRAN EM, VOM SAAL FS: Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environ Health Perspect* **111**: 994-1006, 2003.

WETHERILL YB, AKINGBEMI BT, KANNO J, MCLACHLAN JA, NADAL A, SONNENSCHEIN C, WATSON CS, ZOELLER RT, BELCHER SM: In vitro molecular mechanisms of bisphenol A action. *Reprod Toxicol* **24**: 178-198, 2007.

WOZNIAK AL, BULAYEVA NN, WATSON CS: Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-alpha-mediated Ca2+ fluxes and prolactin release in GH3/B6 pituitary tumor cells. *Environ Health Perspect* **113**: 431-439, 2005.

YE L, ZHAO B, HU G, CHU Y, GE RS: Inhibition of human and rat testicular steroidogenic enzyme activities by bisphenol A. *Toxicol Lett* **207**: 137-142, 2011.

YE L, GUO J, GE RS: Environmental pollutants and hydroxysteroid dehydrogenases. *Vitam Horm* **94**: 349-390, 2014.

ZHANG X, CHANG H, WISEMAN S, HE Y, HIGLEY E, JONES P, WONG CK, AL-KHEDHAIRY A, GIESY JP, HECKER M: Bisphenol A disrupts steroidogenesis in human H295R cells. *Toxicol Sci* **121**: 320-327, 2011.

PŘÍLOHA X

Vitku J, Heracek J, Sosvorova L, Chlupacova T, Hampl R, Hill M, Sobotka V, Bicikova M, Starka L: The impact of bisphenol A and polychlorinated biphenyls on spermatogenesis and steroidogenesis: two biological fluids in men from an infertility clinic. Environment International, odesláno do tisku

The impact of bisphenol A and polychlorinated biphenyls on spermatogenesis and steroidogenesis: two biological fluids in men from an infertility clinic

Jana Vitku[1], Jiri Heracek[2,3], Lucie Sosvorova[1], Tereza Chlupacova[1], Richard Hampl[1], Martin Hill[1], Vladimir Sobotka[4], Marie Bicikova[1], Luboslav Starka[1]

[1] Department of Steroids and Proteofactors, Institute of Endocrinology, Prague, Czech Republic

[2] Department of Urology, First Faculty of Medicine, Charles University in Prague, Czech Republic

[3] Department of Urology, Military University Hospital Prague, Czech Republic

[4] Department of Urology, Third Faculty of Medicine, Charles University in Prague, Czech Republic

Corresponding author:

Jana Vitku (maiden name: Kubatova)

Department of Steroids and Proteofactors, Institute of Endocrinology

Narodni 8,11694 Prague 1, Czech Republic

Telephone number: +420 224 905 238; Email: jvitku@endo.cz

Abstract

Background: In the testis, steroids play an important role in spermatogenesis, the production of semen, and the maintenance of secondary sex characteristics and libido. They may also play a role as a target for substances called endocrine disruptors (EDs). As yet, however, no complex study has been conducted evaluating the relationships between EDs and the steroid spectrum in the plasma and seminal plasma.

Objectives: To shed more light into mechanisms of action of EDs and the effects of Bisphenol A (BPA) and polychlorinated biphenyls (PCBs) on human spermatogenesis and steroidogenesis.

Methods: We determined BPA and 11 steroids in the plasma and seminal plasma of 191 men with different degrees of fertility, using a newly developed liquid-chromatography mass spectrometry method. Concurrently, plasma levels of 6 congeners of PCBs, gonadotropins, selenium, zinc and homocysteine were measured. Partial correlations adjusted for age and BMI were performed to evaluate relationships between these analytes.

Results: Seminal BPA, but not plasma BPA, was negatively associated with sperm concentration (r=-0.212; p=0.005), sperm count (r=-0.178; p=0.018) and morphology (r=-0.156; p=0.049). Divergent and sometimes opposing associations of steroids and BPA were found in both body fluids. The sum of PCB congeners was negatively associated with testosterone, free testosterone, the free androgen index and dihydrotestosterone in plasma.

Conclusion: BPA negatively contributes to the final state of sperm quality. Moreover, our data indicate that BPA influences human gonadal and adrenal steroidogenesis at various steps. Environmental levels of PCBs negatively correlated with androgen levels, but surprisingly without negative effects on sperm quality.

Keywords: bisphenol A, endocrine disruptor, polychlorinated biphenyls, steroid, reproduction, spermatogenesis

1 Introduction

For nearly 30 years there has been ongoing debate regarding the potential harm of substances called endocrine disruptors (EDs). Many studies have dealt with the effects of EDs on various body organs, and despite the difficulties accompanying this research there is emerging evidence for adverse impacts on humans (for review see Diamanti-Kandarakis et al. 2009). The most often discussed EDs include polychlorinated biphenyls (PCBs), dioxins, phthalates and bisphenol A (BPA) because of their persistence in the environment and accumulation in biomass (PCBs and dioxins), as well as their ubiquity in food packaging and other materials (phthalates, BPA). People come into contact with these EDs in the environment and cannot completely avoid exposure in everyday life. The main routes of exposure are through the intake of food, water and air. A further pathway is through dermal contact (Darbre 2015). Regarding reproduction, in 2007 it was concluded based on animal studies that there is substantial evidence of even low dose effects of BPA on reproductive health, specifically a reduction in spermatogenesis (vom Saal et al. 2007).

Human studies investigating the effects of BPA on sperm quality (Goldstone et al. 2014; Knez et al. 2014; Lassen et al. 2014; Li et al. 2011; Meeker et al. 2010a; Mendiola et al. 2010; Vitku et al. 2015b) and reproductive hormone levels (Galloway et al. 2010; Kim et al. 2014; Lassen et al. 2014; Liu et al. 2015; Meeker et al. 2010a; Mendiola et al. 2010; Zhou et al. 2013; Zhuang et al. 2015) have not provided clear results. More studies have been conducted on the impact of PCBs on male reproductive functions, but have also been inconsistent (reviewed in Meeker and Hauser 2010).

It is well known that some substances can adversely affect reproductive functions. Alternatively, other substances such as trace elements selenium (Se) and zinc (Zn) can positively influence these functions (Bleau et al. 1984; Colagar et al. 2009). Potentially beneficial agents include endogenous immunoprotective steroids with antiglucocorticoid effects as dehydroepiandrosterone (DHEA) and its 7-hydroxylated metabolites (Hampl et al. 2003; Chmielewski et al. 2000; Niro et al. 2010)

In 2015, expert panels were convened to estimate the burden and disease costs that can be ascribed to EDs, based on current evidence from the European Union (Trasande et al. 2015). According to relevant studies published by that time,

low epidemiological and strong toxicological evidence for male infertility attributable to phthalate exposure were identified, resulting in a 40-69% probability of causing 618000 additional assisted reproductive technology procedures costing € 4.71 billion annually (Hauser et al. 2015). These estimations focused on exposure to phthalates and polybrominated diphenyl ethers because their impact on reproductive functions was among the best documented in human as well as animal studies (Hauser et al. 2015).

Although the effects of some EDs on reproductive functions are well documented, there remain uncertainties regarding the effects and mechanisms of action of other EDs. The body of literature on animal as well as human studies suggests that EDs can disrupt steroid hormone homeostasis (Clark and Cochrum 2007). Any impairment of the delicate balance in hormone biosynthesis and metabolism can have adverse consequences in the human organism.

In this study we attempted to elucidate to what extent the most well known environmental endocrine disruptors, namely BPA and PCBs, are associated with male reproductive function. The following issues were addressed:

(1) To what degree BPA and PCBs influence spermiologic parameters with respect to different degrees of infertility (from normospermic to azoospermic men).

(2) In connection with this, we were interested in how blood- and seminal plasma levels of the measured analytes correlate with each other.

(3) How the levels of the main reproductive hormones, steroids as well as gonadotropins correlate with the above mentioned EDs. In contrast to numerous studies reported by others, we also measured the main precursors and intermediates, enabling us to map the possible effects of EDs on biosynthetic/metabolic pathways and thus to assess effects on the activities of the responsible enzymes.

(4) To elucidate possible effects on immunity, we also measured 7-oxygenated metabolites of DHEA, which have recently been shown to be present in seminal fluid (Hampl et al. 2000).

(5) In addition, two trace elements involved in the mechanism of reproductive functions, selenium and zinc, along with homocysteine, one of the parameters of

oxidative stress (Forges et al. 2007), were measured to determine whether there are any associations with EDs.

2 Experimental

2.1 Chemicals and reagents

The steroids cortisol, cortisone and DHEA were from Koch-Light Laboratories Ltd. (Colnbrook, Great Britain); 7α-hydroxy-DHEA (7α-OH-DHEA), 7β-hydroxy-DHEA (7β-OH-DHEA), 7-oxo-DHEA, testosterone (T), androstenedione (ADIONE), pregnenolone (PREG), 17-hydroxy-pregnenolone (17-OH-PREG), and deuterated standards of DHEA (D3-DHEA), ADIONE (D7-ADIONE), PREG (D4-PREG), 17-OH-PREG (D3-17-OH-PREG) and dihydrotestosterone (D3-DHT) were from Steraloids (Newport, RI, USA). D4-Cortisol was obtained from CDN isotopes (Ponte-Claire, Canada). D1-7α-OH-DHEA and D1-7-oxo-DHEA were obtained from Betulinines (Stribrna Skalice, Czech Republic). D1-T was synthesized by Sci-Tech (Prague, Czech Republic). DHT, D7-cortisone, 2-hydrazinopyridine, ammonium formate and trifluoroacetic acid were from Sigma-Aldrich (St. Louis, MO, USA). Methanol and water for chromatography were of HPLC grade and were from Merck (Darmstadt, Germany). Diethyl ether was obtained from Lach-Ner, s.r.o. (Neratovice, Czech Republic). The physiological solution (0.9% sodium chloride) was from B. Braun (Melsungen AG, Germany).

2.2 Study group

The studied cohort consisted of 191 Czech men attending the Pronatal Centre of Assisted Reproduction (Prague, CZ) since April 2012. Some of the patients were normospermic men, where the cause of infertility was the female factor, and the others included patients with various degrees of impaired fertility. Each patient underwent a standardized ejaculate examination (spermiogram) according to the World Health Organization (WHO) 2010 criteria. Height and weight were measured, and a basic urological and andrological examination was performed including ultrasonography of the prostate, seminal vesicles and testicles, with no pathological findings observed. Samples of plasma and seminal plasma were collected from each patient. All steps in the sample collection protocol and subsequent processing were carried out using BPA-free glass equipment and stored in glass tubes at -20°C until analysis. For details on how we dealt with possible BPA contamination, see our

previous study (Vitku et al. 2015a). Men were divided into four groups according to their spermiogram. The first group included normospermic men with a normal spermiogram (n=89); oligospermic, asthenospermic and oligoasthenospermic men were included in the second group (n=59); teratospermic, oligoasthenoteratospermic and oligoteratospermic men comprised the third group (n=25); while the fourth group were azoospermic men (n=18). We termed these groups: (1) healthy men, and (2) slightly, (3) moderately and (4) severely infertile men.

The study was performed in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The protocol was approved by the Ethical Committee of the Institute of Endocrinology. Informed and written consent with the use of biological materials for research reasons was obtained from all subjects participating in the project.

2.3 Development and validation of a LC-MS/MS method for determining 10 unconjugated steroids in plasma and 11 unconjugated steroids in seminal plasma

2.3.1 Sample preparation

Our previously published method on selected neuro- and immunomodulatory steroids in blood plasma (Sosvorova et al. 2015) was extended to include the determination of PREG, 17-OH-PREG, cortisol, cortisone, DHEA, 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA, T and ADIONE in plasma and in seminal plasma with minor modifications. In addition, DHT was determined in seminal plasma. Briefly, a sample of plasma (500 µL) or seminal plasma (1000 µL) was spiked with 10 µL of an internal standard (IS) and diluted with 500 µL of physiological solution. Samples were shaken, and a liquid-liquid extraction using diethyl ether (3 mL, 1 min) was performed. Dry residues were derivatized by 100 µL of 2-hydrazinopyridine in methanol with the addition of trifluoroacetic acid (1mg: 5mL: 1.63 µL) according to Higashi et al. (Higashi et al. 2007). The samples were shortly shaken and then sonicated for 15 mins. After evaporating under a gentle stream of nitrogen, samples were redissolved in 100 µL of 5 mM ammonium formate in 60% methanol, of which 50 µL was injected into the liquid chromatograph.

2.3.2 Liquid chromatography/mass spectrometry (LC-MS/MS) of steroids

Chromatography was performed on an ultra-high performance liquid chromatography (UHPLC) Eksigent ultraLC 110 system (Redwood City, CA, USA) equipped with a Kinetex C18 column (100 x 3.0 mm, 2.6µm; Phenomenex, Torrance, CA, USA) and Security Guard ULTRA cartridge system (UHPLC C18 for 3mm ID column; Phenomenex, Torrance, CA, USA). Column temperature was maintained at 50°C and separation was carried out at a flow rate of 0.75 mL/min. Detection of the analytes was performed on an API 3200 mass spectrometer (AB Sciex, Concord, Canada) with electrospray ionization (ESI) probe operating in positive mode. Retention times and transitions with optimized conditions for MS are summarized in Table S1. Analyst 1.6 software was used for system control and data evaluation. More information about the LC-MS/MS conditions can be found in the study of Sosvorova et al. (Sosvorova et al. 2015).

2.3.3 Validation

The analytical method was validated according to the FDA Guidance for Industry (Food and Drug Administration 2001). Validation parameters included (1) selectivity, (2) precision, (3) recovery (analytical accuracy), (4) calibration curve and (5) stability of the analytes in spiked samples. Accuracy, precision and recovery were determined by replicate analysis of 6 samples in 4 different concentrations in plasma, or 3 different concentrations in seminal plasma due to the limited amount of the matrix. Samples were pretreated in the same way as in Section 2.3.1. Pooled plasma, resp. seminal plasma was used as the first concentration. The spiked concentrations in plasma were as follows: 0.28, 1.4 and 3.36 ng/mL for T, PREG and 17-OH-PREG and 0.06, 0.3 and 0.72 ng/mL for ADIONE. Seminal plasma spiked concentrations were as follows: 16 and 80 ng/mL for cortisol; 3.2 and 16 ng/mL for DHEA and cortisone; 0.56 and 2.8 ng/mL for T, PREG and 17-OH-PREG and 0.12 and 0.6 ng/mL for seminal ADIONE, 7α-OH-DHEA, 7β-OH-DHEA and DHT. The results of validation parameters including lower limits of quantifications are given in Table S2 and Table S3 for plasma steroids and seminal steroids, respectively. All results met the FDA guidelines criteria.

Calibration curves were constructed with 1/x weighting and reached good linearity; correlation coefficients for each calibration curve are given in Table S2 and Table S3. A freeze and thaw stability test, short-term temperature stability test, long-

term stability test, stock solution stability test and post-preparative stability test were carried out. All tests showed satisfactory results when comparing instrument responses between freshly prepared samples and corresponding samples after stability testing.

2.4 Additional measurements/methods

BPA and estrogens (estrone-E1, estradiol-E2 and estrone-E3) were measured according to our previously published LC-MS/MS method (Vitku et al. 2015a). Luteinizing hormone (LH), follicle stimulating hormone (FSH) and sex hormone binding globulin (SHBG) were measured using immunoradiometric assay (IRMA) kits from Immunotech (Marseille, France). The immunoassays were processed on a Stratec (France) automatic analyser. Plasma DHT was assessed by a radioimmunoassay method published elsewhere (Hampl et al. 1990).

Se and Zn concentrations in plasma were measured by a commercially available and accredited atomic absorption spectrometry method in Agel Laboratories (Novy Jicin, Czech Republic).

Six congeners of polychlorinated biphenyls (PCB 28, 101, 118, 138, 153, 180) in plasma were determined in the accredited laboratory ALS Czech Republic by gas chromatography-high resolution mass spectrometry (GC-HRMS) (Pardubice, Czech Republic).

Homocysteine was assessed by a previously published method (Husek et al. 2003).The free testosterone (FT) value was calculated according to Vermeulen et al. (Vermeulen et al. 1999). The free androgen index (FAI) was calculated as total testosterone (nmol/L)/SHBG (nmol/L) × 100.

2.5 Statistical analysis

Based on conventional practice, the data that were below the limit of detection (LOD) were replaced by LOD/√2 (Hornung and Reed 1990). All data were subsequently transformed by Box-Cox transformation before further processing due to the non-Gaussian data distribution and non-constant variance (heteroscedasticity) in most variables. Differences between groups were evaluated using one-way ANOVA followed by least square difference (LSD) multiple comparisons. The statistical software Statgraphics Centurion XVI from Statpoint Inc. (Warrenton, VA, USA) was used for data transformations, correlations, ANOVA testing and multiple comparisons. Two-dimensional non-homogeneities (in correlations) were found using

NCSS 2007 (Kaysville, UT, USA) and subsequent partial correlations analyses were performed using the same statistical software.

3 Results

We measured the seminal and plasma concentrations of 11 steroids, reflecting the major biosynthetic pathways, along with seminal and plasma BPA, 6 congeners of PCB in plasma, plasma gonadotropins, Se, Zn and homocysteine, and compared them with parameters of semen quality.

The mean age (± SD) of all participants was 35.8±5.6 years and mean BMI value was 27.2±3.6 kg/m^2. In our study, 55% of men were overweight (BMI 25-30) and 20% were obese (BMI>30). BMI values as well as age did not significantly differ among all the groups of men studied (Table 1).

BPA was detected in 89% of plasma samples and 93% of seminal samples. The most abundant PCB congeners were PCB 180 and PCB 153 (detected in 99 and 100% of samples, respectively) followed by PCB 138 (96% of samples), PCB 118 (56%), PCB 101 (8%) and PCB 28 (5%). Differences were found between BPA levels and levels of the sum of 6 PCB levels and individual PCBs across the groups of men with various degree of fertility (Table 1). Seminal BPA levels increased with increasing severity of infertility. Plasma levels of BPA were significantly higher in the groups of slightly and moderately infertile men in comparison with healthy men and severely infertile men. Levels of the sum of the 6 congeners were lower in the group of moderately infertile men compared with the other groups. Multiple comparisons of PCBs 101 and 28 across groups were not carried out because of the small sample size in these analytes.

Furthermore, significant differences were found in the concentrations of some steroids in plasma as well as seminal plasma across the groups (Table 1). Levels of the rest of measured analytes that were not significantly different between groups are shown in Table S4.

Table 2 shows partial correlations between EDs and spermiogram parameters, adjusted for age and BMI. Seminal BPA negatively correlated with sperm count, concentration and morphology. Plasma BPA was positively associated with non-progressively motile sperms. The sum of PCB congeners was surprisingly positively correlated with sperm concentration and total count.

The next step was to evaluate partial correlations between plasma concentrations of EDs and measured plasma analytes (Table 3). The sum of 6 PCB congeners was positively associated with the stress hormone cortisol. Furthermore, it was negatively associated with T and DHT as well as with markers of androgen status – FT and FAI. Plasma BPA was positively correlated with PREG, 17-OH-PREG and DHEA, which are steroids at the beginning of steroid hormone biosynthesis. Moreover, plasma BPA was negatively associated with DHT and positively associated with E2 and E1.

Correlation analysis of seminal BPA and seminal hormone concentrations revealed different results from that in plasma (Table 4). Contrary to plasma associations, seminal BPA was negatively associated with PREG and 17-OH-PREG. Similarly, seminal BPA concentrations were positively correlated with E2 and E3.

Finally, we investigated how reproductive hormones and BPA in plasma correlate with their corresponding analogues in seminal plasma. Plasma BPA significantly correlated with seminal BPA ($r=0.253$; $p=0.001$). Concerning the reproductive hormones, plasma T significantly correlated with its seminal analogue ($r=0.230$; $p=0.013$), but plasma E2 did not correlate with seminal E2 at all ($r=0.035$; $p=0.698$), nor did plasma DHT with seminal DHT ($r= -0.076$; $p=0.365$). Correlations of other steroids in biological fluids are shown in Table S5.

4 Discussion

In most studies of the effects of EDs on steroids, only urinary EDs were correlated with steroid plasma levels. Only a few papers have investigated plasma or even seminal plasma steroids along with plasma levels of EDs (for review see Hampl et al. 2013).

In our study plasma and seminal BPA correlated slightly with each other, and only seminal BPA was negatively associated with sperm concentration, sperm count and morphology. These results are in accordance with our previous study on a smaller group (Vitku et al. 2015b), though our present study has higher statistical power. Our results showing the negative impact of BPA content in seminal plasma on sperm concentration, sperm count and morphology (see Table 2) are in accordance with some studies (Knez et al. 2014; Li et al. 2011; Meeker et al. 2010b), but are in contrast with others (Goldstone et al. 2014; Lassen et al. 2014; Mendiola et al. 2010). These discrepancies may be due to differences in the biological matrices where BPA

was measured. We only found the association in seminal plasma, but not in blood plasma. All other studies examined the effects of BPA on semen quality in urine, and even in this matrix the results have not been consistent. Levels of BPA in urine seem to rather reflect the excretion rate of BPA, while measurements of unconjugated BPA in the blood likely more accurately reflect its bioavailability in the body. Seminal plasma seems thus to be the optimal body fluid for studying physiology and pathophysiology in the testis. Furthermore, the choice of men – from the general population (Goldstone et al. 2014; Lassen et al. 2014) vs. fertile men (Mendiola et al. 2010) vs. groups recruited from infertility clinics (the present study, Knez et al. 2014; Meeker et al. 2010b) vs. highly exposed populations (Li et al. 2011) may lead to different outcomes. Therefore, more studies are needed to achieve greater consistency.

Associations between PCBs and semen quality were described in detail in the review by Meeker and Hauser, where mainly an inverse association between PCBs and motility appeared to be consistent across studies (Meeker and Hauser 2010). In our study, PCB levels were surprisingly lower in the group of moderately infertile men than in healthy men (see Table 1), and were also positively associated with sperm concentration and total count. Similarly, in the study of Dallinga et al. there were statistically insignificantly ($p=0.06$) higher levels of PCBs in the seminal plasma of men with good semen quality than in men with poor semen quality. They therefore conjecture that the poor semen quality was not caused by exposure to PCBs, but due to other, so far unknown, causes (Dallinga et al. 2002).

Several reports are available on the effects of urinary BPA on reproductive hormone concentrations, but with conflicting results (Galloway et al. 2010; Kim et al. 2014; Lassen et al. 2014; Liu et al. 2015; Meeker et al. 2010a; Mendiola et al. 2010; Zhou et al. 2013; Zhuang et al. 2015). Our finding of a positive association of E2 with BPA in both body fluids is in accordance with 3 studies (Kim et al. 2014; Lassen et al. 2014; Liu et al. 2015). One possible explanation for elevated E2 may be the increased activity or expression of aromatase in the periphery or in the testis. Another explanation for the E2 surplus can be competition with BPA on sulphotransferases and UDP-glucuronosyltransferases; different UDP-glucuronosyltransferase isoforms are considered a major group of enzymes involved in the conjugation of BPA (Trdan Lusin et al. 2012). The *in vitro* study of Zhang et al. on H295R cells supports the latter hypothesis (Zhang et al. 2011). Other reports, however, have not found any

association between BPA and E2 (Galloway et al. 2010; Mendiola et al. 2010). We did not find any association of BPA with T blood- or seminal plasma, in agreement with the already mentioned paper of Mendiola et al (Mendiola et al. 2010).

Conflicting results have also been reported addressing the effect of BPA on plasma gonadotropins: in the present study, gonadotropin levels were not associated with BPA, similarly as in the study of Mendiola et al. (Mendiola et al. 2010). Lassen et al. reported higher LH levels in association with BPA, with no correlation with FSH (Lassen et al. 2014). In contrast, Meeker et al. showed higher FSH levels but not LH levels in association with BPA (Meeker et al. 2010a). Some results from animal studies have reported the suppression of LH and FSH after BPA uptake (Akingbemi et al. 2004; Wisniewski et al. 2015), while others have reported an increase in LH production (Tohei et al. 2001). These inconsistent results only indicate that the eventual effect of BPA on sex steroids does not seem to be controlled at a central level.

Three studies have reported an inverse relationship between BPA and ADIONE (Liu et al. 2015; Zhou et al. 2013; Zhuang et al. 2015), which is not in line with our findings. One possible explanation can be the different degree of exposure: while our subjects were recruited from an infertility clinic, the other studies used participants who were exposed occupationally.

As concerns the effect of PCB, our finding of decreased plasma T in association with PCBs content in blood plasma is in agreement with some other studies (Goncharov et al. 2009; Schell et al. 2014). The same results were obtained from adolescents exposed prenatally to PCBs (Grandjean et al. 2012). Further studies have reported an inverse association with SHBG-bound T (Persky et al. 2001; Turyk et al. 2006) or with FT (Bonde et al. 2008; Richthoff et al. 2003). Some studies have also reported no associations between PCBs and any sort of T (Giwercman et al. 2006; Hagmar et al. 2001).

To the best of our knowledge, our study is the first to focus on the impact of BPA as well as PCBs on the main steps of steroid hormone biosynthesis (Tables 3 and 4). In blood plasma the precursors of reproductive hormones, PREG, 17-OH-PREG and DHEA correlated positively with BPA. The situation in seminal plasma was different, with significant negative correlations found between the former precursors (with the exception of DHEA) and BPA. Given that plasma concentrations of these Δ^5 steroids reflect mainly their production in the adrenals (Sagel et al. 1982),

we can hypothesize that seminal concentrations mainly show levels of Δ^5 steroids of testicular origin. A decrease in the synthesis of reproductive hormone precursors in the testis could be caused (among other things) by an impairment of cholesterol transport to mitochondria by steroid acute regulatory protein (StAR) or the reduced conversion of cholesterol to PREG inside mitochondria.

The ratio of cortisol to cortisone reflects not only adrenal activity, but also the peripheral reduction of cortisone to the active hormone by 11β-hydroxysteroid dehydrogenase type 1 (Vitku et al. 2014). The absence of an effect of PCBs and the only moderate increase by BPA on the ratio in blood but not in seminal plasma, along with slightly increased plasma cortisol levels, suggest only minor effects of EDs on the activity of the latter enzyme. The association of an elevated ADIONE to T ratio in blood plasma with the plasma content of both disruptors may point to decreased activity in some systemic (peripheral) 17β-hydroxysteroid dehydrogenases. Of interest is the negative association of the T/DHT ratio with plasma PCBs, along with their negative effect on systemic testosterone levels. Besides the suppression of systemic testosterone synthesis, this may also indicate the suppression of 5α-reductase activity by PCBs. Finally, BPA, but not PCBs, was negatively associated with the plasma T/E2 ratio. All these results show different effects of the studied EDs, which need not be associated with the effects on the seminal plasma composition.

For the first time, we simultaneously measured 7-oxygenated metabolites of DHEA considered to possess immunomodulatory and immunoprotective properties (Hampl et al. 2003; Chmielewski et al. 2000; Niro et al. 2010). These metabolites are present in the seminal fluid and may counteract the adverse effects of cortisol on testicular steroidogenesis (Hampl et al. 2000). Although we observed an increase of DHEA in association with plasma BPA, no relationship was found for 7-hydroxylated metabolites and selected EDs in either plasma or in seminal plasma.

We also did not find any association between plasma Zn and Se and semen quality, although it is generally accepted that these trace elements have positive effects on spermatogenesis (Ahsan et al. 2014; Camejo et al. 2011; Omu et al. 2015). Finally, we found no relationship between homocysteine and EDs.

5 Conclusion

We conclude that environmental levels of PCB do not impair fertility in the population of Czech men studied here, but we did find a negative association

between the PCBs and androgen levels. On the other hand, elevated seminal levels of BPA were associated with a significant decrease in sperm count, sperm concentration and morphology. Furthermore, our data indicate that BPA reduces the rate of testicular steroidogenesis in the Δ^5 pathway. In contrast, we found a stimulation of adrenal steroidogenesis by BPA. The further disruption of steroidogenesis by BPA possibly occurs at the level of E2 metabolism via the suppression of estradiol catabolism or perhaps by the stimulation of aromatase expression and/or activity.

Acknowledgement: This work was supported by Internal Grant Agency project no. NT/13369 from the Czech Ministry of Health.

Financial interests: The authors declare they have no actual or potential competing financial interests.

6 References

Ahsan U, Kamran Z, Raza I, Ahmad S, Babar W, Riaz MH, et al. 2014. Role of selenium in male reproduction - a review. Anim Reprod Sci 146(1-2): 55-62.

Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP. 2004. Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology 145(2): 592-603.

Bleau G, Lemarbre J, Faucher G, Roberts KD, Chapdelaine A. 1984. Semen selenium and human fertility. Fertil Steril 42(6): 890-894.

Bonde JP, Toft G, Rylander L, Rignell-Hydbom A, Giwercman A, Spano M, et al. 2008. Fertility and markers of male reproductive function in Inuit and European populations spanning large contrasts in blood levels of persistent organochlorines. Environ Health Perspect 116(3): 269-277.

Camejo MI, Abdala L, Vivas-Acevedo G, Lozano-Hernandez R, Angeli-Greaves M, Greaves ED. 2011. Selenium, copper and zinc in seminal plasma of men with varicocele, relationship with seminal parameters. Biol Trace Elem Res 143(3): 1247-1254.

Clark BJ, Cochrum RK. 2007. The steroidogenic acute regulatory protein as a target of endocrine disruption in male reproduction. Drug Metab Rev 39(2-3): 353-370.

Colagar AH, Marzony ET, Chaichi MJ. 2009. Zinc levels in seminal plasma are associated with sperm quality in fertile and infertile men. Nutr Res 29(2): 82-88.

Dallinga JW, Moonen EJ, Dumoulin JC, Evers JL, Geraedts JP, Kleinjans JC. 2002. Decreased human semen quality and organochlorine compounds in blood. Hum Reprod 17(8): 1973-1979.

Darbre PD. 2015. Entry into human tissues. In: Endocrine disruption and human health, Part 1st:Academic Press.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev 30(4): 293-342.

Food and Drug Administration. 2001. Guidance for Industry, Bioanalytical method Validation. Available: http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070107.pdf [accessed 2.11.2015].

Forges T, Monnier-Barbarino P, Alberto JM, Gueant-Rodriguez RM, Daval JL, Gueant JL. 2007. Impact of folate and homocysteine metabolism on human reproductive health. Hum Reprod Update 13(3): 225-238.

Galloway T, Cipelli R, Guralnik J, Ferrucci L, Bandinelli S, Corsi AM, et al. 2010. Daily bisphenol A excretion and associations with sex hormone concentrations: results from the InCHIANTI adult population study. Environ Health Perspect 118(11): 1603-1608.

Giwercman AH, Rignell-Hydbom A, Toft G, Rylander L, Hagmar L, Lindh C, et al. 2006. Reproductive hormone levels in men exposed to persistent organohalogen pollutants: a study of inuit and three European cohorts. Environ Health Perspect 114(9): 1348-1353.

Goldstone AE, Chen Z, Perry MJ, Kannan K, Louis GM. 2014. Urinary bisphenol A and semen quality, the LIFE Study. Reprod Toxicol 51C: 7-13.

Goncharov A, Rej R, Negoita S, Schymura M, Santiago-Rivera A, Morse G, et al. 2009. Lower serum testosterone associated with elevated polychlorinated biphenyl concentrations in Native American men. Environ Health Perspect 117(9): 1454-1460.

Grandjean P, Gronlund C, Kjaer IM, Jensen TK, Sorensen N, Andersson AM, et al. 2012. Reproductive hormone profile and pubertal development in 14-year-old

boys prenatally exposed to polychlorinated biphenyls. Reprod Toxicol 34(4): 498-503.

Hagmar L, Bjork J, Sjodin A, Bergman A, Erfurth EM. 2001. Plasma levels of persistent organohalogens and hormone levels in adult male humans. Arch Environ Health 56(2): 138-143.

Hampl R, Putz Z, Stárka L. 1990. Radioimunologické stanovení dihydrotestosteronu a jeho význam pro laboratorní diagnostiku. Biochem Clin Bohemoslov 19: 157-163.

Hampl R, Hill M, Sterzl I, Starka L. 2000. Immunomodulatory 7-hydroxylated metabolites of dehydroepiandrosterone are present in human semen. J Steroid Biochem Mol Biol 75(4-5): 273-276.

Hampl R, Pohanka M, Hill M, Starka L. 2003. The content of four immunomodulatory steroids and major androgens in human semen. J Steroid Biochem Mol Biol 84(2-3): 307-316.

Hampl R, Kubatova J, Heracek J, Sobotka V, Starka L. 2013. Hormones and endocrine disruptors in human seminal plasma. Endocr Regul 47(3): 149-158.

Hauser R, Skakkebaek NE, Hass U, Toppari J, Juul A, Andersson AM, et al. 2015. Male reproductive disorders, diseases, and costs of exposure to endocrine-disrupting chemicals in the European Union. J Clin Endocrinol Metab 100(4): 1267-1277.

Higashi T, Nishio T, Hayashi N, Shimada K. 2007. Alternative procedure for charged derivatization to enhance detection responses of steroids in electrospray ionization-MS. Chem Pharm Bull (Tokyo) 55(4): 662-665.

Hornung RW, Reed LD. 1990. Estimation of Average Concentration in the Presence of Nondetectable Values. Applied Occupational and Environmental Hygiene 5(1): 46-51.

Husek P, Matucha P, Vrankova A, Simek P. 2003. Simple plasma work-up for a fast chromatographic analysis of homocysteine, cysteine, methionine and aromatic amino acids. J Chromatogr B Analyt Technol Biomed Life Sci 789(2): 311-322.

Chmielewski V, Drupt F, Morfin R. 2000. Dexamethasone-induced apoptosis of mouse thymocytes: prevention by native 7alpha-hydroxysteroids. Immunol Cell Biol 78(3): 238-246.

Kim EJ, Lee D, Chung BC, Pyo H, Lee J. 2014. Association between urinary levels of bisphenol-A and estrogen metabolism in Korean adults. Sci Total Environ 470-471: 1401-1407.

Knez J, Kranvogl R, Breznik BP, Voncina E, Vlaisavljevic V. 2014. Are urinary bisphenol A levels in men related to semen quality and embryo development after medically assisted reproduction? Fertil Steril 101(1): 215-221 e215.

Lassen TH, Frederiksen H, Jensen TK, Petersen JH, Joensen UN, Main KM, et al. 2014. Urinary bisphenol A levels in young men: association with reproductive hormones and semen quality. Environ Health Perspect 122(5): 478-484.

Li DK, Zhou Z, Miao M, He Y, Wang J, Ferber J, et al. 2011. Urine bisphenol-A (BPA) level in relation to semen quality. Fertil Steril 95(2): 625-630 e621-624.

Liu X, Miao M, Zhou Z, Gao E, Chen J, Wang J, et al. 2015. Exposure to bisphenol-A and reproductive hormones among male adults. Environ Toxicol Pharmacol 39(2): 934-941.

Meeker JD, Calafat AM, Hauser R. 2010a. Urinary bisphenol A concentrations in relation to serum thyroid and reproductive hormone levels in men from an infertility clinic. Environ Sci Technol 44(4): 1458-1463.

Meeker JD, Ehrlich S, Toth TL, Wright DL, Calafat AM, Trisini AT, et al. 2010b. Semen quality and sperm DNA damage in relation to urinary bisphenol A among men from an infertility clinic. Reprod Toxicol 30(4): 532-539.

Meeker JD, Hauser R. 2010. Exposure to polychlorinated biphenyls (PCBs) and male reproduction. Syst Biol Reprod Med 56(2): 122-131.

Mendiola J, Jorgensen N, Andersson AM, Calafat AM, Ye X, Redmon JB, et al. 2010. Are environmental levels of bisphenol a associated with reproductive function in fertile men? Environ Health Perspect 118(9): 1286-1291.

Niro S, Hennebert O, Morfin R. 2010. New insights into the protective effects of DHEA1). Horm Mol Biol Clin Investig 4(1): 489-498.

Omu AE, Al-Azemi MK, Al-Maghrebi M, Mathew CT, Omu FE, Kehinde EO, et al. 2015. Molecular basis for the effects of zinc deficiency on spermatogenesis: An experimental study in the Sprague-dawley rat model. Indian J Urol 31(1): 57-64.

Persky V, Turyk M, Anderson HA, Hanrahan LP, Falk C, Steenport DN, et al. 2001. The effects of PCB exposure and fish consumption on endogenous hormones. Environ Health Perspect 109(12): 1275-1283.

Richthoff J, Rylander L, Jonsson BA, Akesson H, Hagmar L, Nilsson-Ehle P, et al. 2003. Serum levels of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) in relation to markers of reproductive function in young males from the general Swedish population. Environ Health Perspect 111(4): 409-413.

Sagel J, Levine JH, Mathur RS, Rosebrock G, Gonzalez J, de Villier C, et al. 1982. Plasma steroid concentrations in patients with hypopituitarism and Kallman's syndrome: effects of testosterone replacement therapy. Clin Endocrinol (Oxf) 17(3): 223-231.

Schell LM, Gallo MV, Deane GD, Nelder KR, DeCaprio AP, Jacobs A. 2014. Relationships of polychlorinated biphenyls and dichlorodiphenyldichloroethylene (p,p'-DDE) with testosterone levels in adolescent males. Environ Health Perspect 122(3): 304-309.

Sosvorova L, Vitku J, Chlupacova T, Mohapl M, Hampl R. 2015. Determination of seven selected neuro- and immunomodulatory steroids in human cerebrospinal fluid and plasma using LC-MS/MS. Steroids 98: 1-8.

Tohei A, Suda S, Taya K, Hashimoto T, Kogo H. 2001. Bisphenol A inhibits testicular functions and increases luteinizing hormone secretion in adult male rats. Exp Biol Med (Maywood) 226(3): 216-221.

Trasande L, Zoeller RT, Hass U, Kortenkamp A, Grandjean P, Myers JP, et al. 2015. Estimating burden and disease costs of exposure to endocrine-disrupting chemicals in the European union. J Clin Endocrinol Metab 100(4): 1245-1255.

Trdan Lusin T, Roskar R, Mrhar A. 2012. Evaluation of bisphenol A glucuronidation according to UGT1A1*28 polymorphism by a new LC-MS/MS assay. Toxicology 292(1): 33-41.

Turyk ME, Anderson HA, Freels S, Chatterton R, Jr., Needham LL, Patterson DG, Jr., et al. 2006. Associations of organochlorines with endogenous hormones in male Great Lakes fish consumers and nonconsumers. Environ Res 102(3): 299-307.

Vermeulen A, Verdonck L, Kaufman JM. 1999. A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 84(10): 3666-3672.

Vitku J, Starka L, Bicikova M, Hill M, Heracek J, Sosvorova L, et al. 2014. Endocrine disruptors and other inhibitors of 11beta-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition. J Steroid Biochem Mol Biol.

Vitku J, Chlupacova T, Sosvorova L, Hampl R, Hill M, Heracek J, et al. 2015a. Development and validation of LC–MS/MS method for quantification of bisphenol A and estrogens in human plasma and seminal fluid. Talanta 140(0): 62-67.

Vitku J, Sosvorova L, Chlupacova T, Hampl R, Hill M, Sobotka V, et al. 2015b. Differences in bisphenol A and estrogen levels in the plasma and seminal plasma of men with different degrees of infertility. Physiological Reseach 64 (Suppl. 2): S303-S311.

vom Saal FS, Akingbemi BT, Belcher SM, Birnbaum LS, Crain DA, Eriksen M, et al. 2007. Chapel Hill bisphenol A expert panel consensus statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. Reprod Toxicol 24(2): 131-138.

Wisniewski P, Romano RM, Kizys MM, Oliveira KC, Kasamatsu T, Giannocco G, et al. 2015. Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis. Toxicology 329: 1-9.

Zhang X, Chang H, Wiseman S, He Y, Higley E, Jones P, et al. 2011. Bisphenol A disrupts steroidogenesis in human H295R cells. Toxicol Sci 121(2): 320-327.

Zhou Q, Miao M, Ran M, Ding L, Bai L, Wu T, et al. 2013. Serum bisphenol-A concentration and sex hormone levels in men. Fertil Steril 100(2): 478-482.

Zhuang W, Wu K, Wang Y, Zhu H, Deng Z, Peng L, et al. 2015. Association of serum bisphenol-A concentration and male reproductive function among exposed workers. Arch Environ Contam Toxicol 68(1): 38-45.

Table 1. Comparisons of analyte concentrations among men with different degrees of infertility

		GROUP 1 (n=89)	**GROUP 2** (n=59)	**GROUP 3** (n=25)	**GROUP 4** (n=18)	p-value	Multiple comparisons
	Age	35.9 (34.8; 37.0)	35.7 (34.3; 37.0)	35.8 (33.8; 37.8)	35.2 (32.9; 37.6)	0.9715	
	BMI	27.7 (26.7;28.8)	26.9 (25.8;28.1)	26.1 (24.9; 27.5)	26.4 (24.8; 28.1)	0.2748	
EDs	Plasma BPA (ng/mL)	0.029 (0.019;0.044)	0.059(0.034;0.106)	0.072 (0.039;0.185)	0.019 (0.008;0.047)	0.0288	1<2,3
	Seminal BPA (ng/mL)	0.075 (0.055;0.100)	0.130 (0.093;0.179)	0.153 (0.091;0.243)	0.148 (0.082;0.250]	0.0180	1<2,3,4
	Σ of 6 PCB congeners (ng/g of plasma)	1.52 (1.35;1.72)	1.30 (1.13;1.51)	1.00 (0.76;1.31)	1.32 (1.00;1.75)	0.0350	3<1
	PCB 180 (ng/g of plasma)	0.66 (0.58;0.76)	0.53 (0.45;0.62]	0.49 (0.36;0.66)	0.57 (0.41;0.77)	0.0984	2<1
	PCB 153 (ng/g of plasma)	0.57 (0.50;0.65)	0.48 (0.41;0.56)	0.35 (0.26;0.47)	0.47 (0.34;0.63)	0.0211	3<1
	PCB 118 (ng/g of plasma)	0.030 (0.25;0.035)	0.026 (0.021;0.033)	0.025 (0.015;0.044)	0.020 (0.012;0.038)	0.5522	
	PCB 138 (ng/g of plasma)	0.223 (0.195;0.256)	0.202 (0.173;0.237)	0.184 (0.137;0.252)	0.206 (0.147;0.295)	0.6410	
PLASMA	LH (IU/L)	2.80 (2.47;3.17)	3.40 (2.92;3.97)	3.25 (2.50;4.25)	5.39 (3.99;7.29)	0.001	1,2,3<4
	FSH (IU/L)	2.94 (2.57;3.37)	3.90 (3.29;4.66)	3.61(2.70;4.90)	10.24 (6.96;15.48)	0.000	1<2,4 2,3<4
	7β-OH-DHEA (ng/mL)	0.149 (0.133;0.166)	0.126 (0.108;0.145)	0.139 (0.110;0.170)	0.104 (0.074;0.137)	0.064	1>4
	Estradiol (ng/mL)	0.017 (0.014;0.021)	0.016 (0.013;0.020)	0.016 (0.011; 0.022)	0.007 (0.004;0.012)	0.016	1,2,3>4
SEMINAL PLASMA	Pregnenolone (ng/mL)	0.198 (0.170;0.231]	0.173 (0.144;0.206)	0.298 (0.229;0.385)	0.182 (0.132;0.249)	0.009	1,2,4,<3
	Cortisol (ng/mL)	4.60 (3.74;5.55)	5.76 (4.67;6.97)	6.88 (5.15; 8.88)	4.88 (3.20;6.93)	0.105	1<3
	DHEA (ng/mL)	1.40 (1.13;1.75)	1.45 (1.14;1.86)	2.40 (1.65;3.57)	1.88 (1.22;2.99)	0.073	1,2<3
	DHT (ng/mL)	0.225 (0.184;0.271)	0.158 (0.121;0.201)	0.135 (0.087;0.198)	0.114 (0.063;0.185)	0.012	1>2,3,4
	Estradiol (ng/mL)	0.0039 (0.0034;0.0045)	0.0041 (0.0034;0.0050)	0.064 (0.0046;0.0097)	0.0050 (0.0036;0.0075)	0.036	1,2<3
	Estrone (ng/mL)	0.0043 (0.0037;0.0050)	0.0047(0.0039;0.0057)	0.0077 (0.0056;0.0109)	0.0047 (0.0034;0.0068)	0.016	1,2,4<3

[a]Data are shown as means and 95.0 percent confidence intervals (in the parentheses) for each group, the levels of significance of the model and multiple comparisons are provided.
Group 1 = normospermic men; Group 2 = oligospermic/asthenospermic/oligoasthenospermic men;
Group 3 = teratospermic/oligoteratospermic/oligoasthenoteratospermic men; Group 4= azoospermic men

Table 2. Partial correlations adjusted for age and BMI between sperm parameters and EDs in plasma and seminal plasma.

Sperm parameters	Σ of 6 PCB congeners in plasma		Plasma BPA		Seminal BPA	
	r	p-value	r	p-value	r	p-value
Concentration (mil/mL)	0.273	**0.000**	-0.119	0.134	-0.212	**0.005**
Total count	0.308	**0.000**	-0.115	0.146	-0.178	**0.018**
Motility	0.069	0.376	0.139	0.079	-0.106	0.164
- Progressively motile sperms	0.112	0.147	0.095	0.233	-0.122	0.108
- Non-progressively motile sperms	-0.064	0.411	0.192	**0.014**	-0.039	0.606
- Immotile sperms	-0.027	0.723	0.071	0.373	-0.037	0.626
Morphology	0.099	0.223	-0.029	0.729	-0.156	**0.049**

[a]The correlation coefficient of partial correlation r is a measure of the strength between variables and the p-value shows statistical significance.

Table 3. Partial correlations adjusted for age and BMI between plasma EDs and measured analytes.

Plasma analyte	Σ of 6 PCB congeners in plasma		Plasma BPA	
	r	p-value	r	p-value
Homocysteine	0.060	0.497	-0.041	0.657
Se	0.078	0.315	-0.001	0.989
Zn	-0.028	0.723	0.163	**0.042**
LH	-0.014	0.862	-0.021	0.792
FSH	0.019	0.812	-0.111	0.166
SHBG	-0.056	0.472	-0.011	0.893
PREG	-0.021	0.786	0.239	**0.002**
17-OH-PREG	0.022	0.778	0.264	**0.001**
Cortisol	0.166	**0.035**	0.099	0.213
Cortisone	0.091	0.250	-0.077	0.331
DHEA	0.050	0.523	0.249	**0.001**
7α-OH-DHEA	-0.128	0.103	0.069	0.386
7β-OH-DHEA	0.021	0.790	0.148	0.062
7-oxo-DHEA	-0.015	0.853	0.012	0.880
ADIONE	-0.037	0.634	0.145	0.069
T	-0.332	**0.000**	-0.044	0.577
FT	-0.337	**0.000**	-0.035	0.660
FAI	-0.260	**0.001**	-0.034	0.673
DHT	-0.191	**0.013**	-0.177	**0.026**
E2	-0.047	0.604	0.357	**0.000**
E1	0.138	0.131	0.286	**0.001**
E3	0.250	**0.006**	0.079	0.372
ratio Cortisol/Cortisone	0.146	0.065	0.176	**0.026**
ratio ADIONE/T	0.221	**0.004**	0.166	**0.037**
ratio T/DHT	-0.320	**0.000**	0.076	0.341
ratio T/E2	-0.097	0.289	-0.372	**0.000**

[a]The correlation coefficient of partial correlations r is a measure of the strength between variables and the p-value shows statistical significance.

Table 4. Partial correlations adjusted for age and BMI between steroids and their ratios and BPA in seminal plasma.

Seminal analyte	Seminal BPA	
	r	p-value
PREG	-0.207	**0.011**
17-OH-PREG	-0.207	**0.015**
Cortisol	0.048	0.565
Cortisone	0.049	0.553
DHEA	-0.125	0.129
7α-OH-DHEA	0.082	0.329
7β-OH-DHEA	0.054	0.514
7-oxo-DHEA	-0.109	0.210
ADIONE	-0.037	0.681
T	0.105	0.266
DHT	-0.075	0.367
E2	0.163	**0.033**
E1	0.061	0.423
E3	0.202	**0.009**
ratio Cortisol/Cortisone	0.042	0.617
ratio ADIONE/T	-0.118	0.220
ratio T/DHT	0.157	0.093
ratio T/E2	0.037	0.695

[a]The correlation coefficient of partial correlation r is a measure of the strength between variables and the p-value shows statistical significance.