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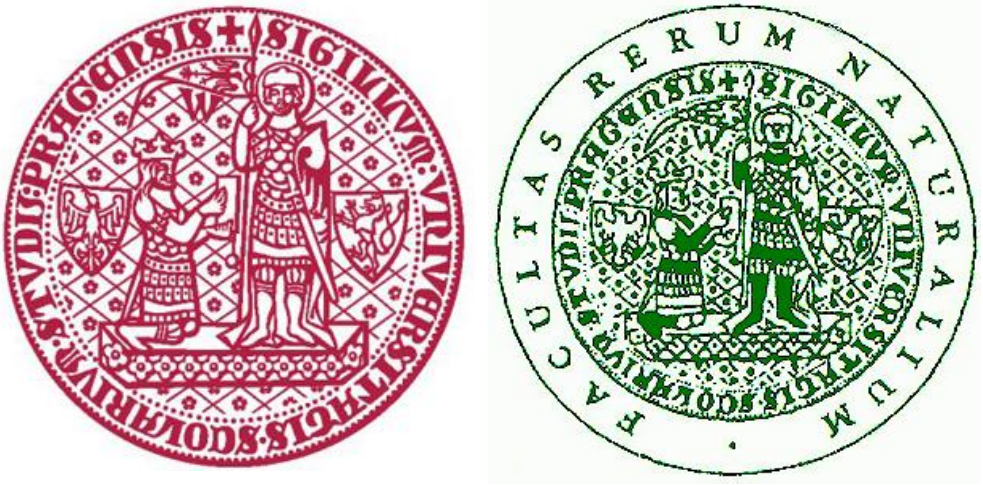

Mgr. Andriy Dorosh

Disertační práce

Vliv vybraných polutantů na savčí organismy *in vivo* a buňky *in vitro* a příprava specifických monoklonálních protilátek k jejich detekci

Effect of selected pollutants on mammalian organisms *in vivo* and cells *in vitro* and preparation of specific monoclonal antibodies for their detection

Školitelka:

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Praha, 2015

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Statement:

I hereby declare that the work presented in this thesis has been conducted independently and without any inappropriate support and that all sources of information, be it experimental or intellectual, are aptly referenced. I also declare that this thesis has not been submitted, either in the same or a different form, to this or any other university for a degree.

V Praze, 8.6.2015

Podpis

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Quod Me Nutrit, Me Destruit

(...)

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Abstract

Environmental pollution and its effect on the living organisms has attracted lots of attention recently. There is a growing body of evidence that we are exposed to environmental pollutants at low concentrations in everyday life. The cells and organisms have tools to identify, neutralize and excrete the majority of the toxic compounds. The most dangerous are those that can escape this process or act at low trace concentrations. Endocrine disruptors (EDs) belong to the latter group.

Endocrine disruptors can be of natural and anthropogenic origin. EDs target corresponding hormonal receptors and can act at low concentrations. A wide family of nuclear receptors recognize steroid hormones. The majority of EDs can pass through the cytoplasmic membrane, use the hydrophobic nature of the receptor-ligand binding, trigger hormone response and change the expression of the sensitive genes. By interfering with estrogen and androgen signaling, EDs can have effect on the whole organism, but the reproductive system is influenced most. In the present work, our aim was to develop the methods for ED detection and monitoring, analyze the estrogenic potency of EDs, and evaluate the effects of natural estrogens and EDs on male reproductive functions, including sperm and testicular physiology and endocrine functions.

First, we prepared a panel of monoclonal antibodies recognizing environmental pollutants and natural estrogens. This allowed fast and reproducible detection of various EDs in environmental water samples. In part of our work we focused on preparation of monoclonal antibodies that recognize surface proteins of the sperm cells interacting with egg envelopes. This allowed us to study in detail the effect of EDs on sperm capacitation and hyperactivation.

Second, we determined the estrogenicity of environmental pollutants *in vitro* and studied the effect of these endocrine disruptors on male fertility and expression of testicular genes during spermatogenesis in a mouse model *in vivo*. We showed that the studied compounds induce changes in testicular gene expression patterns and have a negative effect on the male reproductive system. Our results provide the molecular basis for the underlying mechanisms of EDs action on male reproductive functions during the most susceptible periods of prenatal and pubertal development.

The submitted work has helped us to understand the impact of environmental pollutants on the male reproductive system and sperm maturation.

Souhrn

Znečištění životního prostředí a jeho negativní vliv na živé organismy představuje jeden z největších problémů současné lidské společnosti. Populace je den co den vystavována nízkým koncentracím environmentálních polutantů s potvrzeným či předpokládaným negativním efektem na lidské zdraví. Buňky a organismy si v průběhu evoluce osvojily různé způsoby detekce, neutralizace a exkrece většiny toxických látek vyskytujících se v prostředí. Největší riziko tak představují ty látky, které dokáží detoxikačním systémům organismu uniknout nebo působí i ve velmi nízkých koncentracích. Endokrinní disruptory (EDs) pak často představují právě takový typ látek.

Endokrinní disruptory mohou být přírodního či antropogenního původu, a mohou v nízkých koncentracích ovlivňovat odpovídající hormonálních receptory. Nejčastějším mechanismem jejich účinku je pak vazba na přirozené hormonální receptory. Jaderné steroidní receptory díky své evoluci, rozmanitosti a specifickým afinitním vlastnostem představují častý cíl endokrinních disruptorů, které jsou díky svým chemickým vlastnostem schopné projít přes buněčné membrány. Takovými endokrinními disruptory jsou například ty, které interferují s estrogenní a androgenní hormonální regulací. Po vazbě na příslušné receptory jsou tak tyto látky schopné negativně ovlivňovat všechny orgánové systémy ovlivňované příslušnými hormony, v tomto případě tedy především systém reprodukční.

Naším cílem v předkládané práci tedy bylo vyvinout metody detekci a monitorování přítomnosti a hladiny vybraných endokrinních disruptorů v organismu a v prostředí, analyzovat míru estrogenního účinku vybraných látek a posoudit jejich vliv na samčí reprodukční systém zahrnující funkce spermií, testikulární tkáně a hormonální regulace.

Naším prvním významným výsledkem bylo vytvoření panelu monoklonálních protilátek schopných detekovat látky znečišťující životní prostředí. Pro tento panel byly vybrány hybridomové linie s nejvyšší produkcí specifických protilátek s minimální zkříženou reaktivitou k jednotlivým látkám. Tento panel tak umožňuje rychlou a spolehlivou detekci endokrinních disruptorů ve vzorcích kontaminované vody. V rámci naší práce jsme se take zaměřili na přípravu monoklonálních protilátek, které rozpoznávají povrchové proteiny spermií a reagují s povrchovými proteiny vajíčka. Tento přístup nám umožnil podrobně studovat vliv EDs na kapacitaci a hyperaktivaci spermií.

Druhým významným výsledkem bylo zhodnocení estrogenní aktivity vybraných endokrinních disruptorů *in vitro* a také zhodnocení jejich vlivu na samčí reprodukční orgány a expresi vybraných genů hrajících roli v procesu spermatogeneze *in vivo* na myším modelu. Zde jsme pozorovali signifikantní změny v expresi jednotlivých genů a negativní vliv na některé samčí reprodukční parametry. Naše výsledky poskytují molekulární základ pro pochopení základních mechanismů působení EDs na samčí reprodukční funkce během citlivého období prenatálního a pubertálního vývoje.

Předložená práce přispěla k pochopení vlivu environmentálních polutantů na samčí reprodukční systém a maturaci spermií.

Abbreviations

3β-HSD-1-	3β-hydroxysteroid dehydrogenase/isomerase
ACR -	Acrosin, gene
ACR.2 -	Anti-acrosin, monoclonal antibody
ARE -	Androgen response element
ART -	Assisted reproductive technology
BFR -	Brominated flame retardant
BPA -	Bisphenol A (4, 4'-(propane-2, 2-diyl) diphenol)
BSA -	Bovine serum albumin
b.w. -	Body weight
CD-1 -	Outbred mouse strain (also known as ICR)
CpG -	Region of DNA: cytosine-phosphate-guanine
DES -	Diethylstilbestrol (4, 4'-(3E)-hex-3-ene-3, 4-diyldiphenol; (E)-11, 12-Diethyl-4, 13-stilbenediol)
DHT -	Dihydrotestosterone
DNA -	Deoxyribonucleic acid
E1 -	Estrone ((8R,9S,13S,14S)-3-hydroxy-13-methyl- 6,7,8,9,11,12,13,14,15,16-decahydrocyclopenta[a]phenanthrene-17-one)
E2 -	17β-estradiol ((8R,9S,13S,14S,17S)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,17-diol)
E3 -	Estriol ((16α, 17β)-Estra-1(10), 2, 4-triene-3, 16, 17-triol)
ED -	Endocrine disruptor
EE2 -	Ethinyl estradiol (19-nor-17α-pregna-1, 3, 5(10)-trien-20-yne-3, 17-diol)
ELISA -	Enzyme-linked immunosorbent assay
EPA -	The United States Environmental Protection Agency

ERα - Estrogen receptor α

ERβ - Estrogen receptor β

ERE - Estrogen response element

EU - The European Union

F1 - First generation

FSH - Follicle-stimulating hormone

GAPDHS - Glyceraldehyde-3-Phosphate Dehydrogenase, Spermatogenic, gene

GnRH - Gonadotropin-releasing hormone

HAT - Hypoxanthine-aminopterin-thymidine medium

HBCD - Hexabromocyclododecane (1, 2, 5, 6, 9, 10-Hexabromocyclododecane)

HPG - The hypothalamic–pituitary–gonadal axis

HS - Hypospermatogenesis

KLH - Keyhole limpet hemocyanin

LH - Luteinizing hormone

MA - Maturation arrest

MALDI - Matrix-assisted laser desorption/ionization

MCF7 - Cell line, human breast adenocarcinoma (Michigan Cancer Foundation-7)

miRNA - Micro ribonucleic acid

MND1 - Meiotic nuclear divisions 1 homolog (S. cerevisiae), human gene

MoAb - Monoclonal antibody

MTT - 3-(4, 5-dimethyl thiazolyl-2) 2, 5-diphenyltetrazolium bromide

NOA - Non-obstructive azoospermia

Olfr151 - Olfactory receptor 151, mouse gene

P4 - Progesterone

P450scc - Cytochrome P450 side chain cleavage enzyme

PPIA - Peptidyl-prolyl cis-trans isomerase A gene

qPCR - Quantitative polymerase chain reaction

RAB2A - Member RAS Oncogene Family, protein

Ras - Rat sarcoma, protein family

REACH - EU Regulations on Registration, Evaluation, Authorisation and Restriction of Chemicals

RNA - Ribonucleic acid

RT - Reverse transcription

SCO - Sertoli cell-only syndrome

SPATA22 - Spermatogenesis associated 22, human gene

StAR - Steroidogenic acute regulatory protein

T4 - Thyroxine

TBBPA - Tetrabromobisphenol A (2, 2′, 6, 6′-Tetrabromo-4, 4′-isopropylidenediphenol)

TESE - Testicular sperm extraction

TET - Tetracycline

TET - "Ten-eleven translocation" protein family

TFF1 - Trefoil factor 1 gene

TM4 - Mouse BALB/c testis Sertoli cell line

tRNA - Transfer ribonucleic acid

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling

ZEA - Zearalenone ((3S,11E)-14,16-dihydroxy-3-methyl-3,4,5,6,9,10-hexahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione)

ZP - Zona pellucida

1. Introduction

1.1. Environmental pollutants

Environmental pollution and its effect on the living organisms makes headlines of the newspapers and TV to cover an ecological disaster of a bigger scale that happens from time to time and cannot be foreseen. However, the fact is that we are exposed to environmental pollutants at low concentrations in everyday life. A few popular books increased the awareness of general public that certain chemicals in the environment can exert profound adverse effects on wildlife and human health (Carson, 1962; Colborn, 1996; Krimsky, 2000). The cells and organism have tools to identify, neutralize and excrete the majority of the toxic compounds and the most dangerous are those that can escape this process or act at concentrations which are not able to activate detoxification mechanisms. Endocrine disruptors (EDs) often belong to this group.

Endocrine disruptors can be of natural or anthropogenic origin and have become a great concern recently. The United States Environmental Protection Agency (EPA) estimates that 87,000 chemicals might act as potential EDs, including pesticide chemicals, some commercial chemicals, cosmetic ingredients, food additives, nutritional supplements, mixtures, and environmental contaminants (Vogel, 2005). EDs target corresponding hormonal receptors and can act at low concentrations. One ED can change the basic hormonal regulation and by that trigger the chain of complex signaling processes, resulting in homeostasis shift. A wide family of nuclear receptors recognize steroid hormones. The majority of EDs use this “bug” - the hydrophobic nature of the receptor-ligand binding, can pass through the cytoplasmic membrane, bind to the receptors directly, trigger the hormonal response and change the expression of the sensitive genes. That explains why estrogen and androgen receptors are generally the main targets of EDs. By interfering with estrogen and androgen signaling, EDs can have effect on the whole organism, but the reproductive system is influenced most. Environmental pollutants act not solely by direct mimicking of the natural hormones, but can also interfere with the pathways of hormone production, elimination and storage, as well as change the activity of specific enzymes or ion transport events in certain cases.

Adverse effect of EDs may also be transmitted to the next generations. The exact mechanisms of the transgenerational effect of EDs and the molecular mechanisms of heritability of the adverse changes to further generations are not clear yet. Recent findings suggest the role of epigenetic modifications of histones, DNA and miRNA in the transfer of hormonal system deregulation by EDs to the next generations.

In the next part of the thesis, I will summarize the recent information about the environmental pollutants that we included in our studies based on their widespread presence in the environmental samples, including brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A, mycotoxin zearalenone and tetracycline antibiotics.

1.1.1. Brominated flame retardants

Fire safety regulations demand adding flame retardants to the consumer products to meet strict flammability standards. Three main classes of chemical flame retardants consist of: halogenated hydrocarbons, organophosphorus compounds and inorganic products based on metallic hydroxides. Brominated flame retardants (BFRs) Tetrabromobisphenol A (TBBPA) and Hexabromocyclododecane (HBCD) belong to the class of halogenated hydrocarbons and are efficient in inhibition of combustive reaction in plastics and textile. With a wide use of plastic materials, there are growing concerns about the expansion of BFRs in the environment, their resistance and biodegradation. Due to bromide substituents, some BFRs are considered to be toxic, persistent and bio-accumulative in the environment (Birnbaum and Staskal, 2004). BFRs have been detected in indoor and outdoor air and dust samples (Abdallah et al., 2008), in water, sediments, and in sewage sludge (de Wit, 2002). BFRs can be measured in plants and wildlife throughout the food chain, in human tissues, blood serum, and in breast milk of the exposed occupational populations and in general population (Jenssen et al., 2007; Lignell et al., 2009). Brominated flame retardants may possess endocrine disrupting activity and thus represent a threat to the environment and human health, including infertility problems.

1.1.1.1. TBBPA

Tetrabromobisphenol A (TBBPA, Fig. 1) is the main flame retardant used in printed circuit boards and laminates (BSEF, 2012). The human population is exposed to TBBPA daily as it is used in consumer electronics as well as office and communication equipment. TBBPA is used both as additive and reactive BFR and is added to the plastic polymers at relatively high concentrations 5 to 10 %.

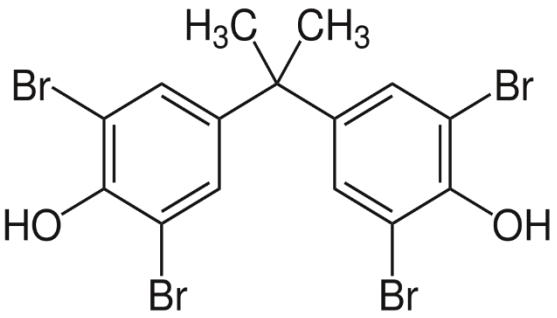

Figure 1. Chemical structure of Tetrabromobisphenol A

The toxicity of TBBPA has been extensively studied on national and international scale due to its high production volumes and potential presence in the environment (Lai et al., 2015). At present, the EU Risk Assessment program identified no risk to the environment and human health when TBBPA is used in reactive applications in printed circuit boards (ECB, 2008; EFSA, 2011b). The major highly reproducible effect of TBBPA was reduction of serum levels of thyroxine (T_4), a form of thyroid hormone. The antagonistic effect of TBBPA on thyroid hormone signaling can be explained by its high-affinity binding to transthyretin (Meerts et al., 2000; Hamers et al., 2006). In this way, TBBPA can compete with T_4 for binding to the transport protein and decrease its concentration in the serum.

It is still unclear whether TBBPA has any estrogenic effect. *In vitro* studies evaluating the direct interaction of TBBPA and estrogen receptor (ER) in E-screen assay, a yeast recombinant model or mammalian cells expressing ER, found that TBBPA either did not bound ER, acted as a weak ER agonist (Olsen et al., 2003; Nakagawa et al., 2007; Lee et al., 2012), or even as an antagonist (Kitamura et al., 2005). In a recent study, Gosavi et al. proposed a possible mimicking of estrogen action by TBBPA in a crystallographic study and proposed a possible mode of TBBPA-protein interaction (Gosavi et al., 2013). Additionally, with the direct relation to male fertility, it was shown that besides endocrine dysregulation, TBBPA at □M concentrations was capable to induce apoptosis of TM4 Sertoli cells by modulation of Ca^{2+} transport proteins and thus disrupt Ca^{2+} homeostasis (Ogunbayo et al., 2008). In a reproductive toxicology study, Tada et al. (Tada et al., 2006) did not observe any changes in measured

reproductive parameters after TBBPA exposure at concentrations 0.01, 0.1 and 1 % in the period from gestation day 0 and postnatally until day 27.

Still, as TBBPA is produced at high volumes topping 200,000 tons/year (BSEF, 2012) and is found mainly in products of daily indoor use, further studies of the long-term exposure to TBBPA are needed

1.1.1.2. HBCD

Hexabromocyclododecane (HBCD, Fig 2), on the other hand, is produced at lower concentrations and products containing HBCD are used mainly outdoor. HBCD is added to the insulation foam boards which are widely used in the construction sector at concentrations 0.5-3 % by weight (BSEF, 2012). HBCD is used only as additive BFR and is not covalently bound to the plastic polymers, and thus can more freely leak from products or waste into the

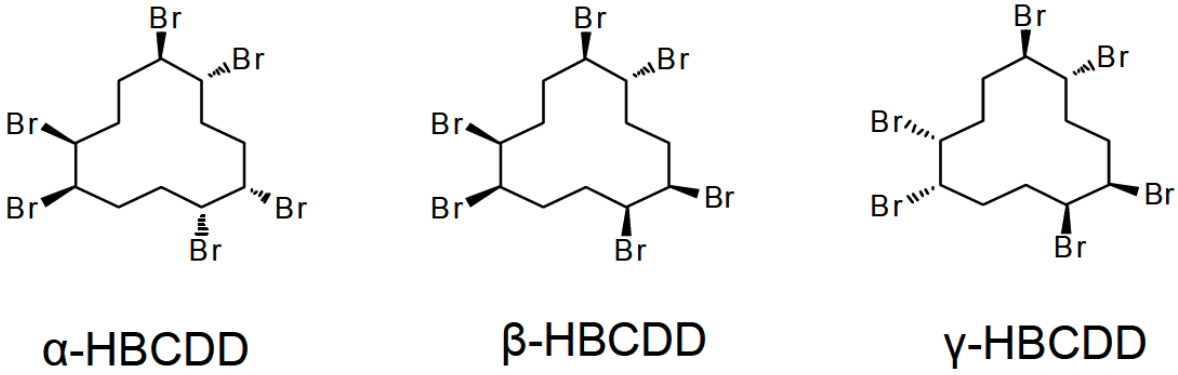

Figure 2. Structure of the three major HBCDD stereoisomers; α-HBCDD, β-HBCDD and γ-HBCDD

environment. This BFR was included in the first tier of studied substances for the risk assessment in the REACH program and considered as "Persistent, Bioaccumulative and Toxic" chemical with several specific risks to the environment.

HBCD is a mixture of three stereoisomers α-, β- and γ-HBCD with α-form being the most persistent in the environmental and food samples (Covaci et al., 2006). The two roots of HBCD contamination are air dust and food; air dust exposure seems to be the main contaminant (Roosens et al., 2009). HBCD was detected in air samples even in Arctic region (de Wit et al., 2010), air dust from indoor and cars at concentrations 1–19 μg/g with highest contamination

in the car microenvironment (Abdallah et al., 2008). The median HBCD concentration in the soil was found to be 0.18 ng/g of dry weight. Detectable levels of HBCD were found in eggs (Covaci et al., 2009; Rawn et al., 2011), fish (van Leeuwen and de Boer, 2008), milk and other food products (Ortiz et al., 2011; Tornkvist et al., 2011). The adipose tissue levels of HBCD in the Czech Republic was at the range of 0.5–7 ng/g of fat and comparable with the other European countries (Pulkrabova et al., 2009).

There was a significant decrease of the testicular weight in male rats exposed to the highest tested concentration of HBCD 100 mg/kg b.w. per day without any further histological abnormalities in the tissue (van der Ven et al., 2009). In another study, male and females rats were continuously exposed to HBCD at different concentrations for two generations (Ema et al., 2008). No reproductive endpoint parameters were changed in male rats after HBCD exposure, except for epididymal sperm counts in the first generation at concentration 150 μg/kg b.w. per day.

To sum up, HBCD is classified to be persistent and bio-accumulative, and further research is needed to evaluate the effect of this BFR on the environment and human health and reproduction in particular.

1.1.2. Zearalenone

Mycotoxin zearalenone (ZEA, Fig. 3) is one of the natural compounds and food contaminants with estrogenic activity, despite its nonsteroidal structure. It is produced by different fungi of the *Fusarium* genus, one of the main grain molds. Humans are exposed to ZEA mainly via oral root with contaminated food and daily exposures vary at 5–100 ng/kg of b.w. per day (EFSA, 2011a). In addition, it was shown recently that ZEA was present in aquatic environment in agricultural regions in seasonal peaks, suggesting its spreading with rain water from the fields (Waskiewicz et al., 2012). ZEA is rapidly absorbed and excreted from the organism. ZEA that remains in the body

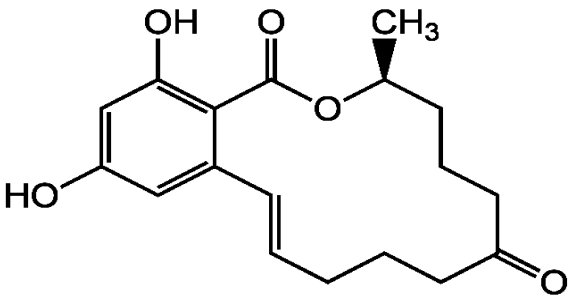

Figure 3. Chemical structure of zearalenone

is targeted to the estrogen-sensitive tissues such as uterus, interstitial cells of the testes and ovarian follicles (Kuiper-Goodman et al., 1987).

Contrary to the previously discussed compounds, ZEA shows a clear estrogenic activity. It can compete with E2 for estrogen receptor binding with binding efficiency 100 to 1000 times lower than natural estrogen and has similar affinity for both forms ERs (Kuiper et al., 1998). ZEA can directly activate transcription from ERE-containing promoters and is, therefore, a potent disruptor of natural estrogen signaling, leading to serious complications in some species, especially pigs (Chang et al., 1979).

Previous results suggest effects of ZEA on sperm motility in boars (Young and King, 1986). Complex studies of the ZEA effect on reproductive parameters were performed on adult albino rats (Ruzsas et al., 1979). Gestational and/or neonatal ZEA administration leads to permanent changes in reproductive organs, disruption of vaginal cycles and disturbed fertility in the offspring.

In an *in vitro* study on mouse Leydig cells, Yang et al. found that ZEA exposure interferes with testosterone production by inhibition of steroidogenic enzymes 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD-1), cytochrome P450 side chain cleavage enzyme (P450scc) and steroidogenic acute regulatory protein (StAR) (Yang et al., 2007a). In another study from the same laboratory, authors described adverse effects of ZEA on the reproductive system of adult male mice *in vivo* (Yang et al., 2007b). Male mice were exposed to intraperitoneal injection of ZEA or α-zearalon at 0, 25, 50, and 75 mg/kg b.w. daily for 7 days, and then mated with sexually mature untreated female mice. The authors showed that in males treated with ZEA, there was a significant decrease in amount of live spermatozoa and increase in number of abnormal spermatozoa. There was a significantly lower pregnancy rate when females were mated with ZEA- or α-zearalon-exposed males. Male mice exposed to ZEA also displayed significant reductions in b.w. and relative epididymis weights. In addition to its estrogenic effect, ZEA seems to interfere with cell signaling and induce germ cell death (Kim et al., 2003; Yuan et al., 2010). Obviously, ZEA displays a lower estrogenic effect than natural E2 or even stronger synthetic estrogen diethylstilbestrol (DES), but still its effect is strong enough to interfere with spermatogenesis by changing basic reproductive endpoints (Filipiak et al., 2009).

1.1.3. Tetracyclines

Unlike zearalenone, tetracycline (TET, Fig. 4) is produced at high quantities intentionally. At present, antibiotics as a wide group of pharmaceutically active chemicals are used not only for acute disease treatment, but also for prevention of disease, enhancement of growth and feed efficiency (Sarmah et al., 2006). Broad-spectrum antibiotics from the tetracycline family bind to 30S ribosomal RNA, preventing the access of tRNA and resulting in inhibition of the synthesis of bacterial proteins (Chopra and Roberts, 2001). Tetracycline is still used in human medicine, but it is produced at high concentration due to its usage in dairy production and for disease prevention. The global use of tetracyclines led to the development of antibiotic-resistant bacteria. Additionally, tetracycline is now detected in environmental samples and can affect non-target organisms. The concentration of TET decreases in effluents from wastewater treatment plants 10 times, but is still detectable at relatively high concentrations 3.6 μg/L (Karthikeyan and Meyer, 2006). TET can now be detected in various aquatic environments at concentrations 0.1 μg/L (Kolpin et al., 2002), but in close proximity to swine farms its concentrations in surface and ground water are tenfold higher (Campagnolo et al., 2002).

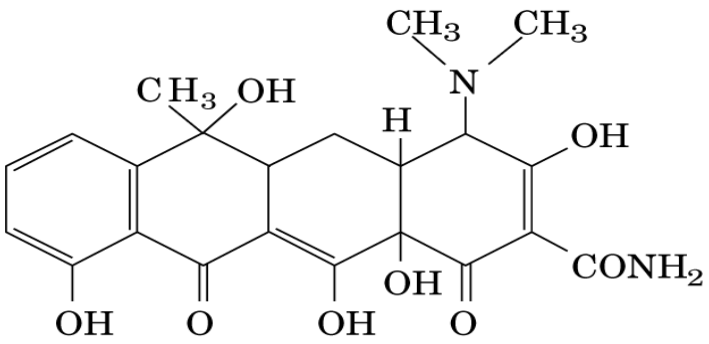

Figure 4. Chemical structure of tetracycline

Tetracycline is well known to reversibly inhibit sperm motility, and therefore it is not advised to undergo assisted reproduction techniques (ART) at least two months after TET treatment. However, there are only few studies on the TET effect on male fertility and reproductive parameters. Kim et al. observed reproductive impairments in model organism *Daphnia magna* after exposure to TET for four consecutive generations (Kim et al., 2012).

In male rats, there was a decrease in testicular and epididymal weight, sperm counts, and increase in oxidative stress immediately after tetracycline exposure (Farombi et al., 2008).

With increased concentrations of tetracycline in water and food (Kemper, 2008), this chemical may also contribute to the decrease of male fertility in mammalian populations.

1.2. Hormonal regulation of male reproductive system

The fundamental role of the endocrine system is to maintain homeostasis of the living organism. This is achieved by a dynamic, refined and coordinated response of tissues and the whole organism to internal or external signals and is mediated by endocrine, neural and immune systems. As the reproductive system in mammals starts to develop at early embryo development and is activated later in puberty, the complex endocrine regulation of this important function is needed. Moreover, sexual reproduction led to the establishment of sex-specific steroid hormones that differently regulate the development of female and male reproductive organs, as well as general morphology, physiology and metabolism. The reproductive system is regulated by hypothalamic-pituitary-gonadal axis (HPG, Fig. 5). This

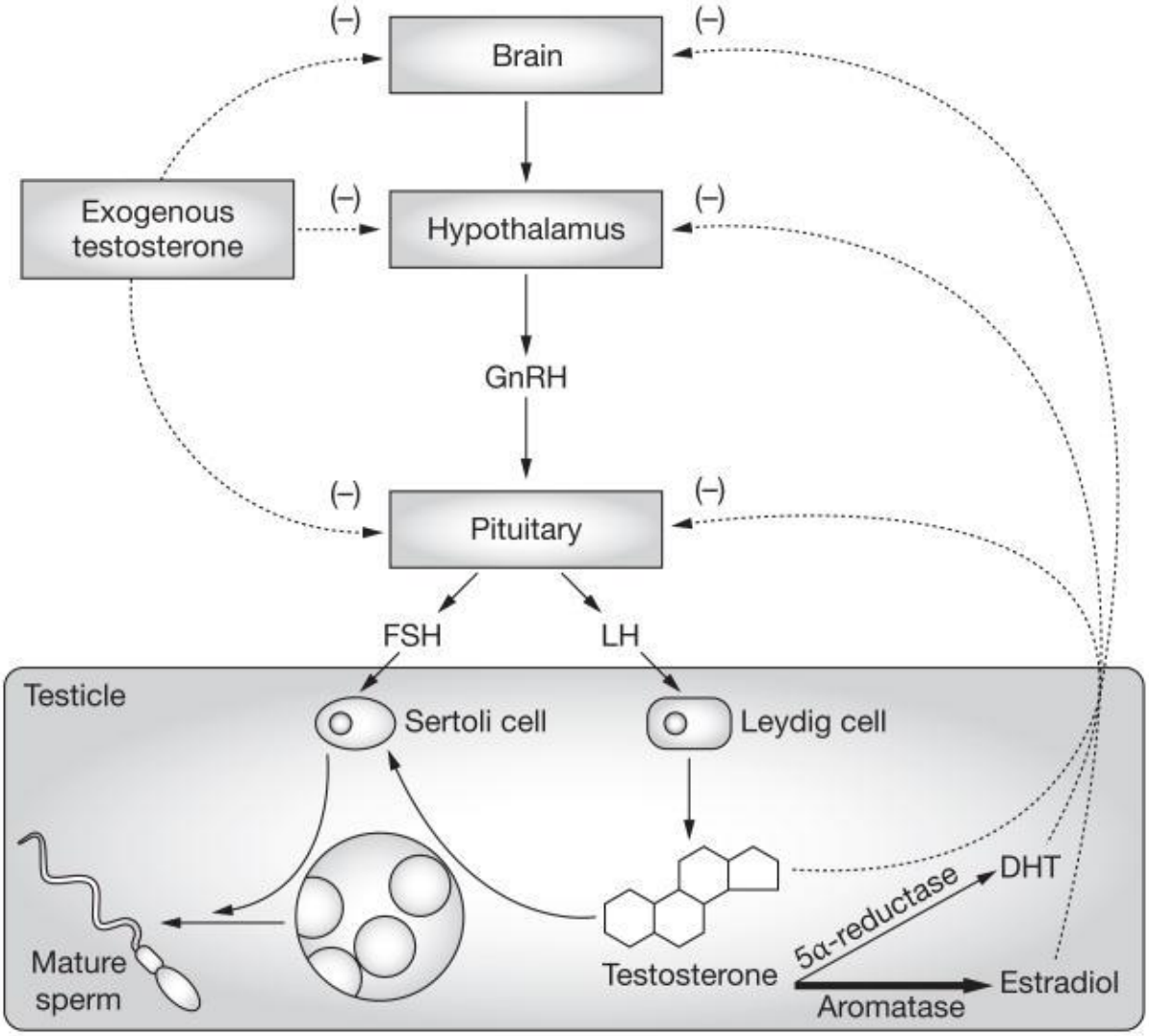

Figure 5. The hypothalamic-pituitary-gonadal axis in men. Abbreviations: DHT, dihydrotestosterone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone (Roth et al., 2008)

results in precise timing, regulation and modulation of the gonadal sex hormone production. In mammalian males, HPG axis consists of GnRH neurons in hypothalamus, gonadotropes in the anterior pituitary, and somatic Sertoli and Leydig cells in the testis. Pulsatile secretion of GnRH from the hypothalamic neurons induces production and secretion of FSH and LH that, in turn, regulate Sertoli and Leydig cell functioning (Terasawa, 1998). In addition, there is a negative feedback loop in which Inhibin B produced by Sertoli cells down-regulates secretion of FSH, and testosterone produced by Leydig cells inhibits LH production (Crowley et al., 1991). The paracrine regulatory system in the testis is established between neighboring Sertoli and Leydig cells, when testosterone from Leydig cells regulates Sertoli cell function and spermatogenesis, and, *vice versa*, Sertoli cells regulate Leydig cell responsiveness to LH stimulation (Sharpe, 2003).

All this complex regulatory network is established at the fetal and early postnatal development. Importantly, the sensitivity and responsiveness of the hypothalamus and pituitary regulation is set up and fine-tuned during this period (Dohler, 1991). Therefore, interference of external hormonal noise would disrupt the correct brain response to sex hormones later in life (Dohler, 1991).

The importance of hormones in sex differentiation has been well described (Wilson et al., 1980). Before sex differentiation the mammalian embryo has the potential to develop male or female reproductive system. The absence of androgen signal will lead to the development of female phenotype, and gonadal production of testosterone will result in the development of generally male phenotype. This is the most vulnerable period of embryo development, when EDs can irreversibly affect the reproductive system and the whole body development.

The general view is that natural estrogens regulate the development and function of the female genital tract, gamete development and maturation, while testosterone is responsible for the male reproductive system development and spermatogenesis. Nevertheless, the serum level of 17β-estradiol (E2) in rat males and men is in the range 20–40 pg/ml (Brewster et al., 1997; Nagata et al., 2001) indicating E2 participation in normal functioning of male reproductive organs (Akingbemi, 2005; Hewitt et al., 2005). Studies on the aromatase (Robertson et al., 1999) or ER knock-out mice suggest that estrogen action is required for fertility in male mice and that the mutation of ER leads to reduced mating frequency, low sperm numbers, and defective sperm function (Eddy et al., 1996).

Classical estrogen receptors, ERα and ERβ, belong to the nuclear receptor superfamily, a group of ligand-dependent transcription factors that regulate the expression of estrogen-sensitive genes. Aromatase and both estrogen receptors were localized in somatic and germ cells in fetal and adult testicular samples (Saunders et al., 1998; van Pelt et al., 1999; Taylor and Al-Azzawi, 2000). The role of estrogens in spermatogenesis and male reproductive system development was indirectly confirmed by adverse effects in the offspring of mothers treated with strong synthetic estrogen diethylstilbestrol (Gill et al., 1976). However, the exact mechanism of E2 action in males is still elusive and under investigation.

The *TFF1* gene was found as one of the first directly induced by ERα upon estrogen signaling (Masiakowski et al., 1982). It is expressed in epithelial mucosa as a small secreted protein with unknown function (Collier et al., 1995). Despite the advances in determining the many estrogen-sensitive up- and down-regulated genes, even potential new ERE (Kwon et al., 2007), *TFF1* is still used as a robust reporter gene to verify the estrogenicity of the studied compounds (Balleine and Clarke, 1999).

1.3. Transgenerational effect of EDs

During risk assessment of specific environmental pollutants on human health, several factors are taken into account. EDs exposure in adulthood is generally compensated by regulation and excretion mechanisms and usually does not result in a significant effect. On the other hand, while the exposure occurs chronically or during most sensitive windows of reproductive and endocrine system development and establishment, permanent changes can occur to the exposed individual and its progeny.

The exact mechanisms of transgenerational inheritance of the changes caused by environmental factors to the subsequent generations are still poorly understood. The majority of environmental pollutants are not teratogenic and cannot produce DNA mutations. The study designs prevent the effect of social inheritance in acquired adverse changes, and therefore, environmentally induced changes might be transferred to next generations, evidently, by epigenetic mechanisms.

In mice, male germ cells generally undergo two distinct active DNA demethylation and imprint erasure processes facilitated by the TET protein family: before the first cell division (Gu et al.,

2011) and while migrating to the germinal ridge (Hackett et al., 2013). The majority of histones in sperm cells are replaced with protamines (Oliva, 2006), erasing histone modification marks, and the trace amounts of miRNA in sperm cells (Gu et al., 2011) can hardly pass environmental information to further generations. Interestingly, unlike in mouse, zebrafish early embryos inherit the sperm methylome, while the oocyte methylation pattern is gradually erased (Jiang et al., 2013).

A recent study in an elegant way described one case of inheritance of the aversion to acetophenone that was associated with negative experience paternally for two generations by epigenetic mechanisms by heritable hypomethylation of two CpGs in an odorant receptor, *Olfr151* (Dias and Ressler, 2014). A number of studies proposed a paternal effect of diet composition and combination of diet and ED bisphenol A on the offspring gene expression changes and metabolic disease (Fullston et al., 2012; Ding et al., 2014; Carone et al., 2015). Another study showed that vinclozolin exposure can lead to deregulation of miRNAs in primordial germ cells in two subsequent generations (Brieno-Enriquez et al., 2015). Previously, it was shown that exposure to the same anti-androgenic ED vinclozolin during the sensitive period of embryonic gonad development also affects the DNA-methylation pattern up to four generations (Anway et al., 2005; Guerrero-Bosagna et al., 2010).

All these findings suggest that while the majority of epigenetic marks and changes acquired during the lifetime are erased by different pathways in the course of embryo and germ cell development, the new organism does not inherit absolute *tabula rasa* or pure genetic information, as some marks of the environmental signals, probably induced by life-threatening or chronic exposures, can pass to the next generations with epigenetic marks. Michael Skinner even proposed a unified theory of evolution, where a Neo-Lamarckian concept can facilitate neo-Darwinian evolution (Skinner, 2015). One of the novel factors to be considered in this theory states: "Environmental exposures at critical developmental windows promote the epigenetic transgenerational inheritance of germline (e.g., sperm) epimutations that alter phenotypic variation" (Skinner, 2015).

2. Aim of the work

In the present work, our aim was to develop methods for EDs monitoring, to analyze the estrogenic potency of EDs, and to evaluate the effects of natural estrogens and EDs on male reproductive functions, including sperm and testicular physiology and endocrine functions.

The selection of specific compounds was based on their widespread concentration in the environment and the potency to affect male reproduction at a low concentration. All chemicals, with the exception of tetracycline, interfere with hormonal regulation and belong to the wide group of endocrine disruptors: bisphenol A, tetrabromobisphenol A, hexabromocyclododecane and zearalenone.

To achieve the main goal of the studies we focused on the following topics:

- Preparation of monoclonal antibodies against estrogens and estrogenic compounds E1, E2, E3, EE2, BPA, TBBPA, HBCD, ZEA and tetracycline;
- Assessment of the estrogenic potency of selected EDs and its comparison with natural estrogens *in vitro*;
- Effect of TBBPA, ZEA and tetracycline on the mammalian male fertility and transgenerational inheritance of the adverse effects;
- Preparation and characterization of monoclonal antibodies recognizing sperm proteins involved in gamete recognition and interaction that will serve as a new tool for determining the effect of EDs on the sperm quality and functioning.

3. Methods

In this section I will describe methods that were used in the first section of the results and have not been published in manuscripts. Other methods employed in the course of the present work can be found in proper sections of the published articles, where they are described in detail.

3.1. Mouse immunization

Adjuvants were used to boost the immune response in a general way and increase the response to the immunizing antigen. Freund's adjuvant was commonly used as the adjuvant, complete in initial immunization and incomplete in following immunizations. The first immunization was performed with 50–200 μg of antigen, the second and third immunization in intervals of 7–10 days and half amount of antigen. During the first two weeks after initial immunization, predominantly antibodies of IgM class could be detected in the serum of the mouse. Three to four immunizations were performed subcutaneously (s.c.) and the final booster immunization was intra-peritoneal (i.p.) 3–4 days before fusion. To investigate whether the immunization elicited the immune response or not, the mouse serum was tested for the presence of antibodies of the desired specificity. Blood was collected from the tail vein about 8 days after the third immunization. The blood was allowed to coagulate for one hour at RT or overnight at 4°C. The blood samples were centrifuged at 1000 rpm for 5 minutes in a bench centrifuge at 4°C. The clear phase, serum, was used to measure the antibody titer. Serum from a non-immunized animal was used as a negative control.

3.2. Cell fusion

General cell fusion is based on the original fusion protocol (Kohler and Milstein, 1975) with small modifications (Gefter et al., 1977). Briefly, Sp2/0 mouse myeloma cells should be in a growth phase and passaged the day prior to the fusion procedure. Usually, one to two 50 ml flasks are sufficient for the fusion. Thawed or freshly isolated spleen cells from the immunized animal and Sp2/0 cells were washed in RPMI medium and mixed in a proportion 5:1 to 2:1,

and in the absolute numbers 5 x 10^6 to 4 x 10^7 cells were used per fusion. Mixed cells were centrifuged at low speed 700 rpm in a tabletop centrifuge to form a loose pellet. The following steps were the most critical for successful fusion. The medium was aspirated as much as possible and the pellet was resuspended gently in the remaining RPMI. Next, 1 ml of warm 37°C 50 % Polyethylene glycol (PEG, m. wt. 1550) was added by slow dropping in the tube wells and gently swirling the tube in 37°C water bath. The cells were centrifuged at 1200 rpm for 3 min and the PEG supernatant was discarded. Eight to 10 ml of warm 37°C RPMI without serum was added over a period of 2 to 3 minutes, again gently swirling the tube to prevent disturbing the cell pellet. The medium was added as gentle drops followed by centrifugation at 1000 rpm for 5 minutes. After removing the medium, clumps of stuck-together cells could be seen at this point, indicating good fusion. Cells were resuspended in fresh RPMI medium at a concentration of 10^6 cells/ml and were allowed to recover for 2 hours in a CO_2 incubator. Finally, feeder cells (macrophages from the mouse peritoneum at 10^6 cells/ml) and 2x concentrated selection medium with HAT was added and the fused cells were plated into 96-well plates at final concentration 0.5 x 10^6 cells/ml or 10^5 cells/ well. Fresh medium was added every 3–4 days and the wells with growing hybridomas were tested against the antigen by an ELISA assay or another screening method. Positive hybridomas were expanded into 24-well plates and subcloned at least two times to ensure production of monoclonal antibodies.

3.3. Cloning of the hybridoma cells

Positive hybrids were cloned two-three times by limiting dilution. Briefly, a selected positive hybrid (50 ml of 1 x 10^4 cells/ml) hybrids were diluted to final concentration of 1 cell/well. Cells received fresh RPMI media with 10% BSA on days 3 and 6 and were monitored for antibody production on days 8–10 with ELISA and immunofluorescence or Western blotting.

4. Results and Discussion

4.1. Monoclonal antibodies recognizing natural estrogens and environmental pollutants

During the first three years of my PhD studies I concentrated on preparation of monoclonal antibodies that can specifically and with high affinity recognize chemicals that are present in the environment at relatively high concentrations, are persistent and can negatively affect the exposed living organisms, including humans.

This part of work was performed in collaboration with the VIDIA spol. s r. o. company that prepared chemically bound conjugates for immunization and screening and performed the final screening and analysis of selected hybridomas (Table 1). As the majority of environmental pollutants are present at nanomolar concentrations and interfere with the endocrine system and hormonal signaling, we decided to also prepare antibodies against natural estrogens. Estradiol, estrone, estriol and ethinylestradiol (part of the hormonal contraception pills) were conjugated with carrier protein KLH to be able to induce immune response and the constructs were used for mouse immunization. Other chemicals such as brominated flame retardants HBCD and TBBPA, component of plastics bisphenol A and antibiotic tetracycline were also conjugated with the carrier protein and used for immune response stimulation. Only about 1–3 % of hybridomas produced antibodies specific to the corresponding compound. Generated antibodies were further analyzed with direct, indirect and sandwich ELISA for specificity and cross-reactivity. The prepared antibodies were used for the detection and monitoring of ED compounds in water samples.

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Table 1. The list of hybridoma cell lines producing antibodies against specified environmental pollutants

Antibody	Clone	Antigen
Anti-estradiol	2H11F11	E2
Anti-estradiol	4E8F8	E2
Anti-estrone	3G1C3	E1
Anti-estriol	5F3A7	E3
Anti-estriol	5F3C4	E3
Anti-estriol	5F3D2	E3
Anti-ethinylestradiol	2D9E12	EE2
Anti-ethinylestradiol	3F10-10	EE2
Anti-tetracycline	3F9E10A4	TET
Anti-tetracycline	3F9E10D2	TET
Anti-bisphenol A	4F10	BPA
Anti-bisphenol A	2B8	BPA
Anti-hexabromocyclododecane	3E6	HBCD
Anti-tetrabromobisphenol A	5E3A9	TBBPA
Anti-tetrabromobisphenol A	5E3D9	TBBPA
Anti-tetrabromobisphenol A	5E3F11	TBBPA

4.2. Assessing the estrogenic potency of brominated flame retardants

Dorosh A, Děd L, Elzeinová F, Pěknicová J. Assessing oestrogenic effects of brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on MCF-7 cells. Folia Biol (Praha). 2011; 57(1):35-9

Tetrabromobisphenol A is produced at the highest volumes and is the main flame retardant used in printed circuit boards and laminates (www.bsef.com). The human population is highly exposed to TBBPA as it is used in consumer electronics as well as office and communication equipment. TBBPA is used as additive and reactive BFR and is added to the plastic polymers at relatively high concentrations. The EU Risk Assessment program identified no risk to the environment when TBBPA is used in reactive applications in printed circuit boards. Hexabromocyclododecane is mainly contained in insulation foam boards, which are widely used in the construction sector (www.bsef.com).

The aim of this work was to evaluate the estrogenic effects of TBBPA and HBCD *in vitro* on MCF-7 cells in comparison to natural estrogens. We used the proliferation test (E-screen assay) in MCF-7 breast cancer cells and qPCR analysis of *TFF1* gene expression to analyze the estrogenicity of the studied compounds. We found that HBCD, but not TBBPA, increased cell proliferation in MCF-7 cells and up-regulated *TFF1* gene expression in a concentration-dependent manner. Anti-estrogen ICI 182,780 reversed the upregulation of the *TFF1* gene by HBCD. We have shown that HBCD displays estrogen-like effects on MCF-7 cells. TBBPA, on the other hand, had no estrogenic effect mediated by the estrogen receptor α in the present model. Nevertheless, it was shown previously that TBBPA or its metabolites can have an adverse effect at the whole organism level, disrupting physiological signaling of thyroid hormones.

4.3. Effect of environmental pollutants on male reproduction in mammals

Zatecka E, Ded L, Elzeinova F, Kubatova A, Dorosh A, Margaryan H, Dostalova P, Peknicova J. Effect of tetrabrombisphenol A on induction of apoptosis in the testes and changes in expression of selected testicular genes in CD1 mice. Reprod Toxicol. 2013 Jan; 35:32-9

Elzeinová F, Pěknicová J, Děd L, Kubátová A, Margaryan H, Dorosh A, Makovický P, Rajmon R. Adverse effect of tetracycline and doxycycline on testicular tissue and sperm parameters in CD1 outbred mice. Exp Toxicol Pathol. 2013 Sep; 65(6):911-7

Zatecka E, Ded L, Elzeinova F, Kubatova A, Dorosh A, Margaryan H, Dostalova P, Korenkova V, Hoskova K, Peknicova J. Effect of zearalenone on reproductive parameters and expression of selected testicular genes in mice. Reprod Toxicol. 2014 Jun; 45:20-30

In the next series of experiments, we evaluated the effect of environmental pollutants on the reproductive parameters of the exposed animals. For an *in vivo* set of studies we selected the substances with high level of worldwide production. Of brominated flame retardants, TBBPA is produced annually at the scale of 200,000 metric tons. The tetracycline group of antibiotics is widely used in veterinary medicine and dairy production as a disease prevention medicine (Sarmah et al., 2006). Zearalenone, on the other hand, is a common contaminating mycotoxin that can be found in cereal crops and has proved estrogenic activity (EFSA, 2011a).

We analyzed the effect of BFR tetrabromobisphenol A on the male reproduction in a multigenerational study. Experimental and control animals of F1 generation were bred in various conditions to enable evaluation of the possible trans-generational effect. Surprisingly, sperm quality and reproductive endpoints were not affected by TBBPA. However, further analysis revealed that the exposed animals had thinner seminiferous epithelium, increased numbers of apoptotic somatic and germ cells in the testes, and decreased amount of epididymal sperm cells. An increased incidence of apoptosis in the testes and changes in the morphometric characteristics of seminiferous tubules were observed in the treated group. In addition, changes

in the expression pattern of selected genes encoding proteins that play an important role during spermatogenesis were observed.

The study of the antibiotics tetracycline family was designed to evaluate their effect during very sensitive pubertal period, when the antibiotics are still used in human medicine for acne treatment. Our results suggest that in addition to the well-known immediate effect of tetracycline on the reproductive system of mammals and sperm motility, some deleterious effects can last long after the antibiotic exposure. Sperm quality analysis, histological examination and TUNEL analysis showed that spermatogenesis in mice is not fully restored even in adulthood after antibiotic exposure during puberty.

Next, zearalenone, especially at the lower, environmentally relevant concentration of 25 ng/kg b.w. per day, negatively influenced the sperm parameters and induced changes in testicular gene expression in exposed CD1 male mice. There was a decrease in sperm concentration (by 40 %) and sperm quality and increase of apoptotic spermatozoa in experimental animals. Based on our gene expression data, we can assume that the decrease in sperm concentration has its origin at the level of spermatogonia and the meiotic phase of spermatogenesis.

4.4. Expression of spermatogenic genes in testicular biopsies from azoospermic patients

Dorosh A, Tepla O, Zatecka E, Ded L, Koci K, Peknicova J. Expression analysis of MND1/GAJ, SPATA22, GAPDHS and ACR genes in testicular biopsies from non-obstructive azoospermia (NOA) patients. Reprod Biol Endocrinol. 2013 May 15; 11:42

Our first intention was to look at the expression of the *TFF1* gene, a reporter gene of estrogen action, in the testicular samples of patients undergoing treatment in the assisted reproduction laboratory to determine possible contribution of EDs to fertility problems. Unfortunately, preliminary results were not confirmed and there was no expression of *TFF1* in testicular tissue. Therefore, we used the available specimens from testicular biopsies of men with non-obstructive azoospermia who underwent TESE to investigate the expression of spermatogenesis-related genes *MND1, SPATA22, GAPDHS* and *ACR*. Non-obstructive azoospermia is a type of azoospermia that is caused by testicular disorders of various origins leading to the disruption of spermatogenesis at some stage and characterized by the absence of sperm cells in the ejaculate opposite to obstructive azoospermia, when sperm cells are produced normally and the absence of sperm in the ejaculate is associated with developmental disorders, sometimes associated with cystic fibrosis and as a result of a vasectomy.

Testicular biopsy specimens were subdivided into three groups according to histological classification of the obstructive azoospermia state: hypospermatogenesis (HS); maturation arrest (MA); and Sertoli cell-only syndrome (SCO). Analysis of the expression of spermatogenic genes in human testes with abnormal spermatogenesis showed different expression patterns in patients from different groups. The fertilization rate for the studied set of patients was 66 % and the pregnancy rate 29 %. In the HS group, the fertilization rate was 72 % and pregnancy rate 32 %, while in the MA group fertilization and pregnancy rates were 54 % and 26 %, respectively. Fertilization rates in relation to the studied genes were uniformly around 70; the pregnancy rates for *ACR* and *GAPDHS* genes were surprisingly low at 6 % and 8 %, respectively.

Our results suggest that expression analysis of genes involved in spermatogenesis can be a fast additional test for determination of the spermatogenesis progress in testicular samples.

4.5. Preparation of monoclonal antibodies against sperm proteins involved in sperm maturation and gamete interaction

Margaryan H*, Dorosh A*, Capkova J, Manaskova-Postlerova P; Philimonenko A, Hozak P, Peknicova J. Characterization and possible function of glyceraldehyde-3-phosphate dehydrogenase-spermatogenic protein GAPDHS in mammalian sperm. Reprod Biol Endocrinol. 2015 Mar 8; 13:15

* These authors contributed equally to the publication

Zigo M, Dorosh A, Pohlová A, Jonáková V, Šulc M, Maňásková-Postlerová P. Panel of monoclonal antibodies to sperm surface proteins as a tool for monitoring localization and identification of sperm-zona pellucida receptors. Cell Tissue Res. 2015 Mar; 359(3):895-908

In this section we concentrated on the preparation and characterization of antibodies involved in the sperm-zona pellucida binding and its regulation.

In the first study, we initially characterized the sperm protein recognized by monoclonal antibody Hs-8 that was prepared by immunization of BALB/c mice with human ejaculated sperms. Previously, Hs-8 antibody was successfully used to determine the quality of sperm cells and their ability to mature and fertilize the eggs (Peknicova et al., 2002; Tepla et al., 2006). In the immunofluorescence test, Hs-8 antibody recognized the protein localized in the acrosomal part of the sperm head and in the principal piece of the sperm flagellum in the human, boar and mouse spermatozoa. Hs-8 labelled the 45 kDa protein in the extract of human sperm, and with sequence analysis it was identified as GAPDHS, one of the ten enzymes of the glycolytic pathway, which are highly conserved and present in nearly all living organisms. In male germ cells undergoing spermatogenesis, at least some somatic glycolytic enzymes are replaced with sperm-specific isoforms. The first enzyme of the second glycolytic phase, glyceraldehyde 3-phosphate dehydrogenase-spermatogenic (GAPDHS), is encoded by a different gene than somatic GAPDH and was shown to be essential for energy production and sperm motility. Inhibition of the GAPDHS enzyme with chlorinated compound α-chlorohydrine had been a promising alternative for hormonal contraception long before somatic and testicular isoforms were identified, but severe side effects found during clinical trials halted

the research in this direction (Mohri et al., 1975). We hypothesized that GAPDHS, like its somatic counterpart, might be involved in other cellular processes in addition to glycolysis. To confirm that, functional analysis of GAPDHS found in the sperm acrosome was performed using the boar sperm-zona pellucida binding assay. We tested the effect of both Hs-8 and commercial anti-GAPDHS antibodies on the sperm-zona pellucida binding; anti-P4 (anti-progesterone antibody) and ACR.2 (anti-acrosin antibody) were used as negative and positive controls, respectively. There was a four- to five-fold decrease in the number of bound sperm cells to the oocyte when ACR.2, Hs-8, or anti-GAPDHS antibodies were present in the incubation medium. Anti-P4 antibody had no effect on the sperm-oocyte binding. The outcome of the *in vitro* sperm-oocyte binding assay suggests involvement of the GAPDHS protein in the secondary sperm-zona pellucida binding.

To sum up, GAPDHS is, in the first place, a sperm-specific glycolytic enzyme involved in energy production during spermatogenesis and sperm motility. In addition, it seems to exert an additional function in the sperm head as well. We confirmed GAPDHS localization in the apical part of the sperm head in addition to the principal piece of the flagellum. In an indirect binding assay, we showed that anti-GAPDHS antibodies interfere with the secondary sperm-oocyte binding

In second study, we characterized the candidate proteins that are involved in the sperm-zona pellucida binding in a boar model. First, we raised a panel of monoclonal antibodies against the purified protein pool from the apical part of the boar capacitated sperm surface. Three proteins with molecular masses that were shown to interact with ZP glycoproteins in a Far Western Blot were determined by MALDI analysis. The first protein recognized by 4C7 antibody was identified to be an acrosin precursor (45 kDa); the 5C5 antibody recognized RAB-2A (24 and 27 kDa), and the 1H9 antibody recognized the P47 protein. Proacrosin participates in secondary sperm-zona pellucida binding and is known to be present in the acrosomal matrix and inner acrosome membrane, and only recently was shown to be present on the surface of ejaculated and capacitated sperm cells (Kongmanas et al., 2014). P47 protein belongs to the lactadherin protein family and participates in the primary sperm-zona pellucida binding (Ensslin et al., 1998). RAB-2A, the member of a subgroup of the Ras superfamily proteins, was demonstrated on the sperm surface for the first time and its role in the sperm-zona pellucida is not known. Previously, it was shown that Rab-2A participates in acrosome biogenesis and growth during spermatogenesis (Mountjoy et al., 2008).

Antibodies recognizing sperm proteins involved in the gamete interaction will be useful tools in the future studies on the effect of EDs on the sperm maturation, capacitation process and sperm-oocyte crosstalk.

5. Conclusion

In the present work we used a complex approach to study the effect of environmental pollutants on the male reproductive system. First, a panel of monoclonal antibodies recognizing EDs was generated as a tool to monitor their concentration in the environment with reasonable sensitivity and no cross-reaction.

In an *in vitro* study, we showed that brominated flame retardant HBCD, but not TBBPA, displayed an estrogenic effect on the MCF-7 cell model using both proliferation assay and reporter gene *TFF1* that contains ERE in its promoter and is highly sensitive to the estrogen signal.

In a series of reproductive toxicology studies, we investigated the adverse effects of environmental pollutants TBBPA, ZEA, doxycycline and TET on male reproductive parameters. In addition to basic reproductive endpoints, changes in gene expression and protein distribution on sperm cells were analyzed.

We studied the expression of genes involved in different stages of spermatogenesis in testicular samples from patients undergoing treatment in the assisted reproduction laboratory. This could help identify the level of spermatogenesis in testicular tissues. However, additional analysis is needed to investigate whether infertility problems were associated with the exposure to environmental factors or are of genetic origin.

As estrogen signaling participates in sperm maturation and capacitation, we generated monoclonal antibodies against sperm surface proteins as necessary tools to study the effect of EDs not only on male reproductive organs and germ cell development, but also on sperm capacitation and gamete interaction.

To sum up, the findings presented in this PhD thesis bring important information to the growing body of evidence that environmental pollutants can influence mammalian organisms and especially their reproductive parameters via different mechanisms.

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7. Supplements

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Characterization and possible function of glyceraldehyde-3-phosphate dehydrogenase-spermatogenic protein GAPDHS in mammalian sperm

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Abstract

Background: Sperm proteins are important for the sperm cell function in fertilization. Some of them are involved in the binding of sperm to the egg. We characterized the acrosomal sperm protein detected by a monoclonal antibody (MoAb) (Hs-8) that was prepared in our laboratory by immunization of BALB/c mice with human ejaculated sperms and we tested the possible role of this protein in the binding assay.

Methods: Indirect immunofluorescence and immunogold labelling, gel electrophoresis, Western blotting and protein sequencing were used for Hs-8 antigen characterization. Functional analysis of GAPDHS from the sperm acrosome was performed in the boar model using sperm/zona pellucida binding assay.

Results: Monoclonal antibody Hs-8 is an anti-human sperm antibody that cross-reacts with the Hs-8-related protein in spermatozoa of other mammalian species (boar, mouse). In the immunofluorescence test, Hs-8 antibody recognized the protein localized in the acrosomal part of the sperm head and in the principal piece of the sperm flagellum. In immunoblotting test, MoAb Hs-8 labelled a protein of 45 kDa in the extract of human sperm. Sequence analysis identified protein Hs-8 as GAPDHS (glyceraldehyde 3-phosphate dehydrohenase-spermatogenic). For this reason, commercial mouse anti-GAPDHS MoAb was applied in control tests. Both antibodies showed similar staining patterns in immunofluorescence tests, in electron microscopy and in immunoblot analysis. Moreover, both Hs-8 and anti-GAPDHS antibodies blocked sperm/zona pellucida binding.

Conclusion: GAPDHS is a sperm-specific glycolytic enzyme involved in energy production during spermatogenesis and sperm motility; its role in the sperm head is unknown. In this study, we identified the antigen with Hs8 antibody and confirmed its localization in the apical part of the sperm head in addition to the principal piece of the flagellum. In an indirect binding assay, we confirmed the potential role of GAPDHS as a binding protein that is involved in the secondary sperm/oocyte binding.

Keywords: Monoclonal antibodies, Spermatozoa, GAPDHS, Immunolabelling, Transmission electron microscopy, *in vitro* sperm/zona pellucida binding assay

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Background

Sperm proteins are important for the structure and function of these specific, highly differentiated cells. The function of these proteins turned out to be involved in energy production (23%), transcription, protein synthesis, transport, folding and turnover (23%), cell cycle, apoptosis and oxidative stress (10%), signal transduction (8%), cytoskeleton, flagella and cell movement (10%), cell recognition (7%), metabolism (6%) binding of sperm to the oocyte and other unknown functions (11%) [1-5].

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is a glycolytic enzyme catalysing oxidative phosphorylation of glyceraldehyde-3-phosphate, yielding 1,3-diphosphoglycerate, which is used by phosphoglycerate kinase to produce ATP. In addition, glycolysis results in production of pyruvate, which is a substrate for mitochondria. Therefore, the enzyme plays a significant role in cellular metabolism and energy regulation. In mammals, there are two isoenzymes encoded by two different genes: somatic isoform (GAPDH) and sperm isoform (GAPDHS). GAPDH is present in all tissues of the organism and is localized predominantly in the cell cytoplasm. After breaking of cells, GAPDH is easily extracted with aqueous solutions. The enzyme consists of four identical subunits of 36 kDa. Each subunit of human muscle GAPDH consists of 335 amino acid residues (UniProtKB/Swiss-Prot ID: G3P_HUMAN). The central role in the catalysis is played by the cysteine residue of the active site (Cys 152). The enzyme can be easily affected by different oxidants, resulting in oxidation of the essential cysteine residues with complete loss of the dehydrogenase activity [6-8].

Glyceraldehyde-3-phosphate dehydrogenase-S, GAPDHS, is highly conserved between species, showing 94% identity between rat and mouse and 87% identity between rat and human. Within a particular species, GAPDHS also shows significant sequence similarity to its GAPDH paralog (70%, 71% and 68% for the rat, mouse, and human, respectively). Previous studies of the sperm-specific isoform of the glycolytic enzyme GAPDH – GAPDHS – show a high conservation level of the protein sequence between the two proteins, with the exception of the extra N-terminal part of GAPDHS. This proline-rich part confers a change in biochemical properties of the enzyme. While GAPDH is an abundant cytoplasmic protein, highly soluble and easy to purify and crystallize, the sperm GAPDHS protein becomes highly insoluble, slowly migrating in the gel, and numerous attempts to determine the crystal structure of the whole protein failed due to its properties [9-11]. Its crystal structure without the N-terminal part was found and shows high similarity to the somatic enzyme. As this glycolytic enzyme became a promising target for male non-hormonal contraception long before it was known that the spermatozoa possess the product from the separate gene [7], the structure of the complete protein and its difference from the somatic isoform is crucial for efficient drug design [12].

In mature sperm cells, energy metabolism enzymes are spatially separated, with mitochondria located in the midpiece and glycolytic enzymes in the principal piece of the tail [13]. Previous studies of GAPDHS revealed its localization in the principal piece of the sperm tail [14,15]. Notably, N-terminal polyproline extension has been proposed to facilitate an association and tight binding of the protein to the fibrous sheath in the principal piece [16].

Other proteins, namely at the cell surface, play a role in sperm/oocyte recognition (primary sperm/oocyte binding) [4], and intra-acrosomal proteins participate in the secondary sperm/oocyte binding [17]. The first step in characterization of the cellular functions is identification of the proteins involved. Understanding the physiological role of certain proteins determines their use in further research, diagnostic applications and development of specific treatments.

In our previous work, we tested the effect of selected antibodies on the sperm/egg binding in the swine model. One of the tested antibodies was monoclonal antibody Hs-8. This antibody had no influence on the cell surface sperm proteins and sperm/oocyte binding after incubation with the sperm. On the other hand, when the antibody was present in the medium during sperm and oocyte co-incubation, after acrosome reaction it was bound to the acrosome proteins and prevented the sperm/egg binding. We have confirmed that the antibody binds to the acrosomal proteins that might be involved in the secondary binding of sperm to the egg [5].

In the present work, we identified GAPDHS as a specific antigen for Hs-8 antibody in the sperm cells and confirmed that both Hs-8 and commercial GAPDHS antibodies block the sperm/zona pellucida binding *in vitro*.

Methods

Chemicals

Analytical-grade chemicals were utilized. BSA (albumin bovine fraction V, pH 7.0), Immobilon-P membrane, Tween 20, Triton X-100 and Coomassie Brilliant Blue (CBB) R-250 were obtained from Serva (Germany). Gelatin, dithiothreitol (DTT), iodoacetamide, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS) were from Sigma (Prague, Czech Republic); thiourea, urea and IPG buffer (pH 3–10) were purchased from Amersham Biosciences (Uppsala, Sweden). Protein standards were from Bio-Rad (Hercules, CA, USA), chemiluminescent substrate (SuperSignal, Pierce) was obtained from Rockford (USA) and VectaShield mounting medium for fluorescence with DAPI H-1200 from Vector Laboratories, (Burlingame, CA, USA).

Cells

Human ejaculated spermatozoa were obtained from the Iscare IVF Ltd., and Pronatal Ltd., Prague. All sperm donors gave their written informed consent with donating the sperm ejaculates for the purposes of the research project. The study was also approved by the institutional review board at the Institute of Biotechnology. The evaluation of semen density, motility and morphology was carried out in compliance with World Health Organization standards [18]. Boar ejaculates were obtained from the Insemination Station Klimetice (Czech Republic). Mouse spermatozoa were obtained from the proximal fifth region of the left and right cauda epididymis of BALB/c mice (AnLab Ltd., Prague, Czech Republic). All procedures were approved by the Committee for Animal Welfare and Protection.

Antibodies

Monoclonal antibodies designated Hs-8 and ACR.2 were prepared in our laboratory by immunization of BALB/c mice with human ejaculated sperms. Hyperimmune spleen cells were fused with Sp2/0 myeloma cells. Positive clones were selected by ELISA with human sperm extracts and by indirect immunofluorescence with human spermatozoa. The immunization procedure and hybridoma technology were described in detail by Peknicova et al. [19]. Monoclonal antibody Hs-8 reacted with intra-acrosomal human and boar sperms [5]. Commercial mouse monoclonal antibody (MoAb) IgG1 to recombinant GAPDHS (ab57062, Abcam, UK) was used in verification tests. Monoclonal anti- progesterone (P4) was raised in our laboratory by immunization of mice with P4-BSA conjugate and selected with P4-OVA conjugate. Goat anti-mouse IgG (γ-chain specific) and IgM (μ-chain specific) antibodies conjugated with fluorescein isothiocyanate (FITC) (Sigma, Prague, Czech Republic), goat anti-mouse IgG-PE antibody (Santa Cruz Biotechnology, USA), horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (GAM/Px) from Bio-Rad (Prague, Czech Republic) were used as secondary antibodies.

Indirect immunofluorescence and co-localization

Human and boar ejaculated sperms and mouse epididymal spermatozoa were suspended into phosphate-buffered saline (PBS, pH 7.4) for 5 min at 37°C and centrifuged for 15 min at 200× *g*. Next, sperm cells were washed twice and diluted in PBS to a final concentration of 50×10^6 cells ml^{-1}. Small drops of the cell suspension were smeared (10 μl) onto glass slides and the remaining spermatozoa were used for protein extraction.

Dried smears were fixed with acetone (10 min, room temperature), rinsed with PBS, and after blocking with bovine serum albumin (1 h, 2% bovine serum albumin in PBS) incubated for 1 h with Hs-8 MoAb (undiluted hybridoma supernatant, immunoglobulin (Ig) concentration < 20 μg ml^{-1}) and with mouse monoclonal antibody to recombinant GAPDHS for 60 min at 37°C. For appropriate controls, smears were incubated with nonspecific monoclonal antibody, with the supernatant of myeloma cells, and with the FITC-conjugate only. After washes with PBS, the smears were incubated with secondary antibodies. In case of MoAb Hs-8, the secondary FITC-conjugated goat anti-mouse IgM (diluted 1:128 in PBS) was used and for anti-GAPDHS, FITC-conjugated goat anti-mouse IgG (diluted 1:64 in PBS) was used and incubated for 60 min at 37°C, washed in PBS, rinsed with distilled water and mounted in Vectashield medium. For double immunolabelling experiments, glass slides with the sperm smears were fixed and blocked as described above and incubated with MoAb Hs-8 and FITC-conjugated anti-IgM secondary antibody. The same slides were incubated subsequently with the commercial anti-GAPDHS antibody and goat anti-mouse IgG-PE secondary antibody.

Samples (200 sperm cells per slide) were evaluated and viewed with a Nikon Eclipse E400 fluorescent microscope equipped with 40x Nikon Plan 40/0.65 lenses and photographed with a CCD camera VDS1300 (Vosskühler, Osnabrück, Germany) with the aid of the NIS elements AR imaging software (Laboratory Imaging, Prague, Czech Republic).

Immunoelectron microscopy

Human fresh ejaculated sperm was separated by SupraSperm System (ORIGIO, Denmark), washed three times in PBS and fixed on ice for 30 min at 0°C in 3% paraformaldehyde and 0.1% glutaraldehyde in Sörensen buffer (SB; 0.1 M sodium/potassium phosphate buffer, pH 7.3), washed twice with SB (10 min each). Cells were then dehydrated in a series of ethanol solutions with increasing concentration of ethanol. Ethanol was then replaced in two steps by LR White resin (Polysciences Inc., Warrington, USA), and the resin was polymerized for two days at +4°C under UV light. After cutting 80 nm sections, nonspecific labelling was blocked by preincubation with 10% normal goat serum (British BioCell International Ltd., Cardiff, UK), 1% BSA and 0.1% Tween 20 in PBS for 30 min at room temperature (RT). For double immunogold labelling experiments, the sections were simultaneously incubated with Hs-8 and commercial anti-GAPDHS primary antibodies, washed three times in PBT (0.005% Tween 20 in PBS), and then incubated with 6 nm gold-conjugated Goat Anti-Mouse IgG (Fcγ fragment specific) and 12 nm gold-conjugated Goat Anti-Mouse IgM (μ chain specific) secondary antibodies (Jackson Immuno Research Laboratories, inc., USA), washed again twice in PBT, then twice in bi-distilled water, and air dried. Finally, sections were contrasted with a saturated solution of uranyl acetate in water

(4 min) and observed in electron microscope Morgagni 268 (FEI, Czech Republic) operated at 80 kV. Control incubations without primary antibodies proved that the signal was highly specific and that there was no cross-reactivity in case of multiple labelling.

Isoelectric focusing, SDS-PAGE and Western blotting

Washed spermatozoa were diluted in PBS, centrifuged at 10000x *g* for 5 min, and extraction buffer was added to the sperm pellet (100 μl of the extraction buffer per 10 × 10^7 cells): SDS (2% m/v SDS (sodium dodecyl sulphate), 1% v/v glycerol, 50 mM Tris buffer titrated with HCl to pH 6.8) or RHB (rehydration buffer: 7 M urea, 2 M thiourea, 4% CHAPS,1% Triton X-100, 20 mM Tris). SDS extracts were vortexed, boiled in water bath for 3 min, cooled to 4°C and centrifuged (23,000x g, 5 min, 4°C). RHB extracts were incubated for 1 h at RT and centrifuged (23,000x *g*, 5 min, RT). The solubilized samples were divided into aliquots and stored at −80°C for electrophoresis and subsequent analysis. The pure GAPDHS recombinant protein (PO1) from Abnova (Oxford, UK) was directly used for the analysis.

The sperm samples were mixed with RHB, 2% (v/v) IPG buffer (3–10), 1% DTT and 0.005% bromophenol blue (added to the final concentration) and incubated for 1 h at RT. The solubilized proteins (200 μg of proteins in total volume 180 μl) were placed onto 7-cm, pI range 3–10, linear strips and rehydrated overnight (according to manufacturer's instructions). Strips were focused at RT. For 2D electrophoresis, 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used.

SDS-PAGE was carried out in 12% slab gels. The protein samples were mixed with reducing SDS sample buffer (50 mM Tris buffer titrated with HCl to pH 6.8, 1% v/v glycerol, 2% m/v SDS, 5% v/v 2-mercaptoethanol, 0.002% m/v bromophenol blue) and boiled for 3 min. Samples of proteins of the sperm extract (total quantity 25 μg) and recombinant protein GAPDHS (5 μg) were applied to the wells. Electrophoretic separation was carried out at constant current 16 mA for each gel in Tris-glycine electrophoretic buffer, pH 8.3 (25 mM Tris, 192 mM glycine), with 0.1% m/v SDS at 4°C. The relative molecular masses of the separated proteins were estimated using prestained Precision Plus Protein Standards run in parallel.

Further, the proteins were transferred onto Immobilon-P membrane for immunodetection. Electroblotting was carried out for 1.5 h at 500 mA and 4°C in TRIS-glycine buffer (pH 9.6) with 20% (v/v) methanol. The membrane was blocked with 5% (w/v) gelatin in PBS-T (0.05% Tween 20 in PBS) at 4°C overnight. After washing with PBS-T, the membrane was incubated with supernatant MoAb Hs-8 (1:15 dilution in 1% gelatin-PBS-T) or anti-GAPDHS MoAb (0.1 μg/μl) at RT for 1 h. Following a washing step, incubation with (GAM/Px) (diluted 1:3000 in 1% gelatin-PBS-T) was peformed for 1 h at RT. After washing, the membrane was developed in the dark with chemiluminescent substrate (SuperSignal) to visualize the corresponding bands.

CBB staining and sequence analysis

Gels intended for mass spectrometric analysis were stained with Coomassie Brilliant Blue (CBB) for visualization of all separated proteins. After SDS-PAGE, the gels were incubated at RT in a solution containing CBB (0.25% CBB R-250, 7% CH3COOH, 50% ethanol) for 1 h. After incubation with CBB, the gels were destained in 35% ethanol with 10% CH3COOH until the background disappeared and the separated proteins were clearly visible.

The mass of individual peptides obtained after tryptic digestion of Hs-8-detected protein was determined by the MALDI method. Mass spectra of peptides were measured using a MALDI-Time-of-Flight (MALDI-TOF) mass spectrometer, a peptide map was established and mass spectra were searched against the database using Profound software. Mass spectrometer BIFLEX II (Bruker-Franzen, Bremen, Germany) was equipped with a nitrogen laser (337 nm) and a gridless delayed extraction ion source. Ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. The spectrum was calibrated internally using the monoisotopic $[M+ H]^+$ ions of trypsin autoproteolytic products. A saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% ACN/0.2% trifluoroacetic acid was used as a MALDI matrix. One microliter of matrix solution was mixed with 1 ml of the sample on the target and the droplet was allowed to dry at ambient temperature.

Isolation and culture of porcine oocytes

Porcine oocytes were recovered from fresh ovaries about 3 h after slaughter by puncturing and aspirating of 3 to 5 mm follicles. Oocytes were collected in BSA-PBS medium, placed in MPM (modified Parker medium) under paraffin oil, and incubated for 48 h at 37°C under 5% CO_2 to complete maturation. After culturing, oocytes were transferred in a number of 40 to 50 pieces in 0.5 ml of 3 M DMSO in culture medium for 10 min at 4°C, and the tubes with oocytes were placed into liquid nitrogen vapours until use. On the day of binding assay, the tubes with frozen oocytes were thawed at 37°C, 5% CO_2. Then the oocytes were washed three times in BSA-PBS, transferred to the drops of medium and overlaid with paraffin oil.

In vitro sperm-zona pellucida-binding assay

We examined the effect of monoclonal antibodies (Hs-8 and anti GAPDHS) during sperm/oocyte co-incubation.

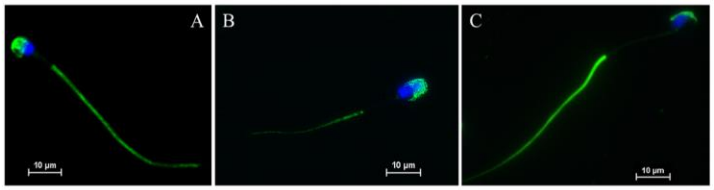

Figure 1 Cellular localization of the Hs-8 antigen in mammalian spermatozoa. IF of human **(A)**, boar **(B)** and mouse **(C)** spermatozoa with Hs-8 monoclonal antibody (*green)*, nuclear staining (*blue*). Bar: 10 µm.

The oocytes (10–20) were transferred to 120 µl droplets containing monoclonal antibodies Hs-8 and GAPDHS, respectively, or monoclonal antibody against progesterone, ACR.2, or MPM medium only (control groups). Capacitated spermatozoa (50 µl) were added to the control and experimental groups of oocytes. After 30 min, co-incubation was stopped by adding 50 µl of 10% NaN_3. Oocytes with bound spermatozoa were washed twice and fixed in 2.5% paraformaldehyde. After washing, the fixed oocytes were stained with Hoechst 33342 (Sigma, Prague) solution (0.3 mg Hoechst /10 ml BSA-PBS), rinsed twice and mounted in a very small droplet of 50% glycerol in PBS (pH 9.0), and Hoechst-labelled spermatozoa attached per oocyte were counted under a fluorescent microscope.

Statistical analysis

Experimental data were analysed and plotted using STATISTICA 6.0. and GraphPad Prism 5.04. Twenty oocytes were analysed per each control and experimental group (N = 20) in each experiment; the total number of analysed oocytes was 100. Sperm samples were obtained from two boars. The differences in the number of bound sperm cells among control and experimental groups were analysed by Kruskal-Wallis test, the post-hoc analysis was performed by Dunn's multiple comparison test. The p value equal to or lower than 0.05 was considered to be significant, *p value ≤0.05 (**p ≤0.01 and ***p ≤0.001).

Results

Indirect immunofluorescence and localization of Hs-8 protein on mammalian sperm

We analysed the interaction of the mouse monoclonal Hs-8 antibody with human, boar and mouse sperm cells. The staining pattern was similar in all these species. The signal was present in the acrosomal part of the sperm head and in the principal piece of the sperm flagellum, while it was absent in the periacrosomal part and mid-piece (Figures 1A,B,C). The recognized epitope on the sperm cells seems to be conserved in all the studied species.

Biochemical characterization of the Hs-8 target antigen

To identify and characterize the antigen that was recognized by MoAb Hs-8, the lysate of human sperm cells was separated with 2D gel electrophoresis followed by protein detection. The signal was obtained at basic pI 8 and 50 kDa (Figure 2). Next, the corresponding protein dot was excised from the polyacrylamide gel and

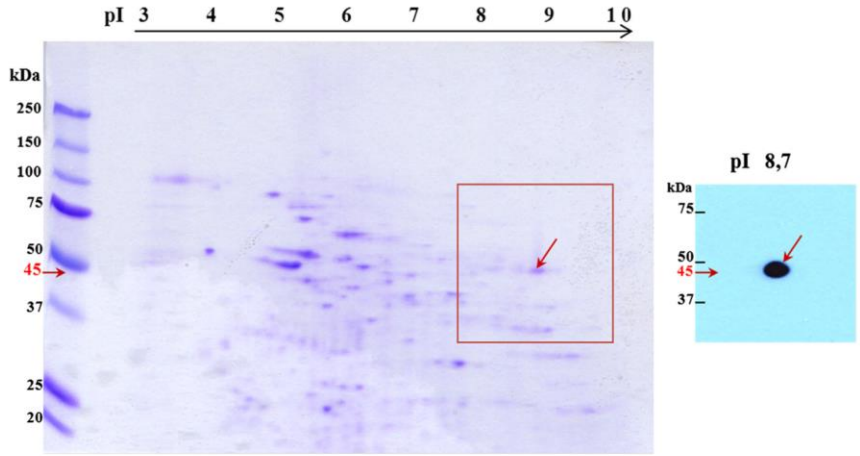

Figure 2 Localization of the Hs-8 target antigen on the two-dimensional map of human sperm proteins. 2D-PAGE (12%), Western blotting and immunodetection of human sperm extract with MoAb Hs-8, which labelled a protein of 45 kDa.

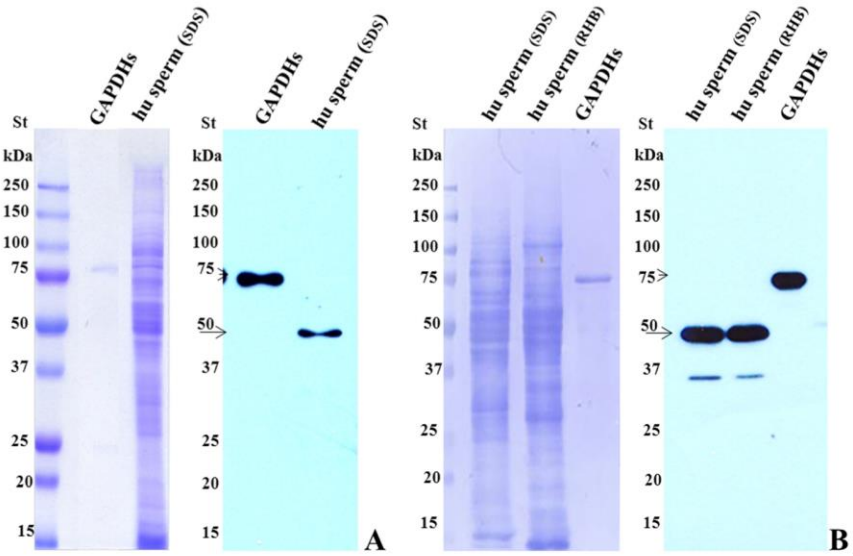

Figure 3 Biochemical confirmation of the Hs-8 target specificity. Immunodetection of human sperm extracts and purified recombinant GAPDHS-GST tagged protein with MoAb Hs-8 **(A)** and with commercial mouse monoclonal antibody against GAPDHS **(B)**. Arrows indicate the GAPDH protein labelled with anti-GAPDHS antibody. St – protein standard, hu sperm – human sperm cell lysate, GAPDHs – recombinant purified GAPDHS protein, hu sperm (SDS) – SDS extract of human sperm cells, hu sperm (RHB) – human sperm cell proteins extracted with RHB buffer.

sequenced. Sequence analysis identified the target protein of the MoAb Hs-8 as GAPDHS (glyceraldehyde 3-phosphate dehydrohenase-spermatogenic), nominal mass (Mr): 44.83 kDa; pI value: 8.7. The number of peptides searched was 24 and the number of those that matched the identified protein was 18. Total sequence coverage was 48% and estimated Z-score: 2.32.

The specificity of Hs-8 antibody and commercial mouse anti-GAPDHS antibody to GAPDHS protein

To confirm that the antigen recognized by Hs-8 is indeed the GAPDHS protein, commercial antibody anti-GAPDHS and recombinant GAPDHS protein conjugated with GST tag were used for analysis. Both Hs-8 (Figure 3A) and anti-GAPDHS (Figure 3B) antibodies labelled bands of the same molecular weight (50 kDa) in immunoblot analysis. At the same time, recombinant protein with MW of 75 kDa was recognized by both commercial anti-GAPDHS and Hs-8 (Figures 3A and B, respectively). Next, we looked whether the data from biochemical analysis could be confirmed by indirect immunofluorescense. Both home-made Hs-8 and commercial anti-GAPDHS are mouse antibodies, but of different isotypes, and double staining could be performed using differently conjugated secondary antibodies against either IgG or IgM. Double immunolabelling with both antibodies demonstrated strong co-localization of the signal in both principal piece and apical part of the sperm head, suggesting that the antibodies recognize the same antigen (Figure 4).

Ultrastructural localization of the GAPDHS protein

Ultrastructural analysis of double immunogold labelling with both antibodies was performed with electron microscopy for precise localization of GAPDHS in the sperm head. It showed that the Hs-8 antigen (12 nm gold particles, arrowheads) and GAPDHS protein (6 nm

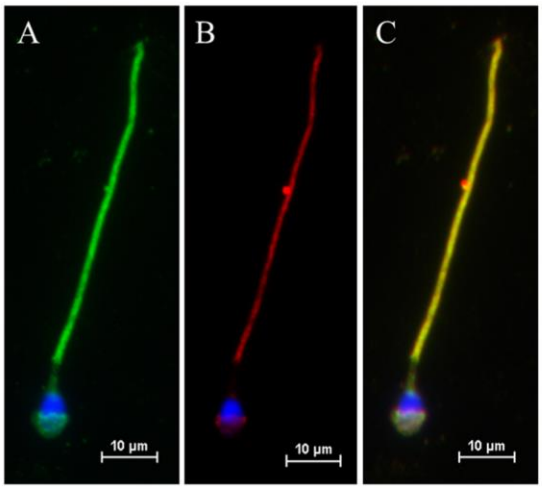

Figure 4 Co-localization of Hs-8 and anti-GAPDHS antigens in the human sperm cells. A. Hs-8 staining of the sperm cell. Indirect immunofluorescence of human spermatozoa with MoAb Hs-8 (green). **B**. Cellular localization of GAPDHS protein detected with anti-GAPDHS antibody (red). **C**. Merge. Yellow colour represents co-localization of the Hs-8 antigen with GAPDHS signal in the sperm head and tail. Bar: 10 μm.

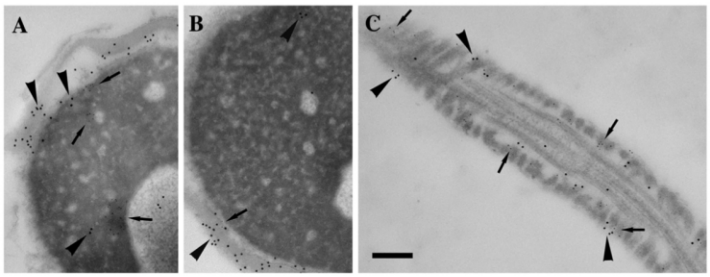

Figure 5 Ultrastructural localization of Hs-8 and GAPDHS antigens. Immunogold labelling of the sections of the sperm head **(A, B)** and principal piece of the tail **(C)** with Hs-8 (12 nm gold particles, arrowheads) and anti-GAPDHS (6 nm gold particles, arrows) antibodies. The signal after staining with either antibody showed a similar pattern and the gold particles appeared in the fibrous sheath of the principal piece in the tail **(C)** and acrosomal part of the sperm head **(A, B)**. Bar: 200 nm.

gold particles, arrows) are localized in close proximity in the nuclear and acrosomal regions of the sperm head (Figures 5A and B, respectively) and in the sperm tail (Figure 5C).

Effect of monoclonal antibodies Hs-8 and GAPDHS on sperm/zona pellucida-binding

During co-incubation of capacitated spermatozoa with oocytes in the presence of monoclonal antibodies Hs-8 and GAPDHS, and ACR.2 as positive control, the sperm binding was strongly reduced. The presence of Hs-8 and GAPDHS decreased the number of spermatozoa to 18% and 21%, respectively, compared to the control group (without antibody). In the positive control with monoclonal antibody ACR.2 (against acrosin), the number of bound spermatozoa was reduced by 25%, see also Figure 6.

Discussion

Among useful tools for studying sperm cell proteins are monoclonal antibodies. If the antibody detects a protein with important functions, it can be used in diagnostics [20-22]. In our laboratory, we prepared a panel of monoclonal antibodies against intra-acrosomal proteins that can be used for determination of sperm quality by assessing the acrosomal status. Using these antibodies, we assessed the quality of mouse spermatozoa [23-25]. We also used monoclonal antibodies against sperm proteins for evaluation of the sperm quality in experimentally induced pathology in mice using endocrine disruptors or anti-androgens [26,5,27] and in humans for evaluation of sperm pathology [28]. Monoclonal antibody Hs-8 against human intra-acrosomal sperm protein is part of this panel. This antibody was used as a tool for evaluation of the human sperm quality and reproductive potential after intra-cytoplasmic sperm injection of various human semen samples [29,28] and is part of the commercial SpermFlow Kit (Exbio, Czech Republic). For this reason, we wanted to further characterize and identify its target protein.

Biochemical and sequence analysis revealed that this sperm antigen is the testes-specific glycolytic enzyme GAPDHS. Slower migration of the protein band at ca 50 kDa instead of the calculated 45 kDa in SDS-PAGE was shown to be due to the proline-rich N-terminal part of the protein and indirectly confirms that GAPDHS is the target antigen for Hs-8 antibody. Indeed, Hs-8 antibody readily interacted with purified GAPDHS-GST on western blot in a similar way as commercial anti-GAPDHS antibody. Another proof of the Hs-8 specificity was indirect immunofluorescent and immunogold labelling of the sperm cells with anti-GAPDHS and Hs-8 antibodies. In both cases, the signal appeared in the principal piece and acrosomal part of the sperm cells.

As the GAPDHS presence in the principal piece of the sperm tail was in line with previous studies, GAPDHS

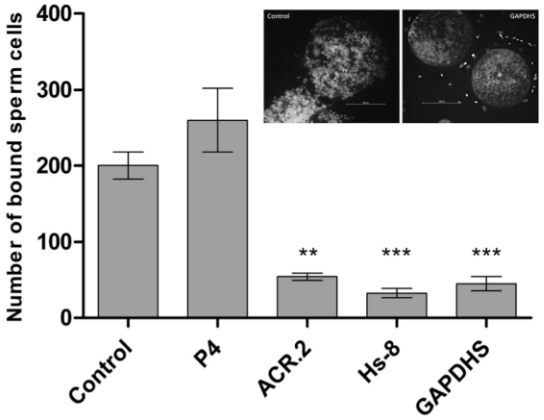

Figure 6 Boar sperm-zona pellucida binding in the presence of Hs-8 and anti-GAPDHS antibodies during sperm-oocyte co-incubation. The number of bound sperm cells on the oocyte after co-incubation with no antibody (Control), and various antibodies: anti-progesterone (P4), acrosin (ACR.2), Hs-8 and anti-GAPDHS (GAPDHS). Two representative images showing sperm oocyte binding with anti-GAPDHS (right side) and no antibody (left side) are displayed in the chart area. * - p value ≤0.05, ** - p ≤0.01 and *** - p ≤0.001).

localization to the acrosomal part of the sperm head was surprising. Nevertheless, there was already some evidence that at least in boar, GAPDHS is localized not only in the principal piece of the sperm flagellum, but also in the acrosomal part [30]. Moreover, GAPDHS protein was found in the protein pool from the human sperm cell nucleus [31]. This supports the idea that GAPDHS, similarly as its somatic isoform GAPDH, might play some additional function in the sperm cells. It is worth noting that bacterial glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GapA-1) might be involved in adhesion of *Neisseria meningitidis* to human cells [32].

In our previous work, we already tested the effect of Hs-8 antibody on the sperm/egg binding. This antibody had no influence on the cell surface sperm proteins and sperm/oocyte binding after pre-incubation with the sperm prior to the binding assay. However, when the antibody was present in the medium during sperm and oocyte co-incubation, it prevented the sperm/egg binding. It seems that Hs-8 binds to the acrosomal proteins after acrosome reaction and blocks the secondary binding of sperm to the egg [5]. In this study, we tested the effect of both Hs-8 and GAPDHS, while anti-P4 (anti-progesterone) and ACR.2 (anti-acrosin) were used as negative and positive controls, respectively. There was a four- to five-fold decrease in the number of bound sperm cells to the oocyte when ACR.2, Hs-8, or anti-GAPDHS antibodies were present in the incubation medium. Anti-P4 had no effect on the sperm/oocyte binding. The outcome of the *in vitro* sperm-zona binding assay suggests involvement of the GAPDHS protein in the secondary sperm/zona pellucida binding.

Still, further analysis of the exact mechanism of GAPDHS interaction with the oocyte and the finding of its interacting partner are needed in the future to confirm that the GAPDHS enzyme plays a role in mammalian gamete interaction.

Conclusions

To sum up, we characterized the Hs-8 protein and identified it as the intra-acrosomal sperm protein GAPDHS. We have also found that its probable role in the sperm head is the secondary binding of sperm to the egg.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HM contributed to the overall design of the study and performed the immunofluorescence assays, AD carried out biochemical analysis and helped to draft the manuscript, JC assisted in data analysis and edited the manuscript, PP participated in the molecular analysis, AP and PH conducted electron microscopy, JP supervised the study, participated in the design and coordination of the study and edited the manuscript for submission. All authors read and approved the final manuscript.

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REGULAR ARTICLE

Panel of monoclonal antibodies to sperm surface proteins as a tool for monitoring localization and identification of sperm–*zona pellucida* receptors

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Abstract Primary binding of the sperm to the *zona pellucida* (ZP) is one of the many steps necessary for successful fertilization. Sperm bind ZP by means of membrane receptors which recognize carbohydrate moieties on ZP glycoproteins according to a well-defined sequential process. Primary binding receptors, many of which have been disclosed in various mammals, are localized throughout the acrosomal region of the sperm surface. A panel of monoclonal antibodies against proteins from the sperm surface was prepared. Antibodies were screened by immunofluorescence for protein localization and Western blotting. Proteins localized on the sperm head and simultaneously detected by Western blotting were further studied in terms of immunolocalization in reproductive tissues and fluids, binding to ZP, immunoprecipitation and sequencing. Of 17 prepared antibodies, 8 recognized proteins localized on the sperm head and also detected proteins of interest by Western blotting. Only three other antibodies recognized proteins that also coincided in binding to ZP. These three antibodies were used for immunoprecipitation, and further protein sequencing of immunoprecipitates revealed that these antibodies distinguished acrosin precursor, RAB-2A protein, and lactadherin P47. This is not the first time we have detected acrosin on the surface of ejaculated and capacitated sperm. However, to our knowledge, this is the first time RAB-2A has been detected on the sperm surface. Lactadherin P47 has already been characterized and its physiological function in reproduction has been proposed.

Keywords Sperm surface proteins · Monoclonal antibodies against sperm surface proteins · *Zona pellucida*-binding receptors · RAB-2A, Lactadherin P47

Introduction

The pig is one of the most studied animal models in the pursuit of elucidating the processes taking place during mammalian fertilization. The fundamental mechanism of gamete recognition seems to be conserved throughout evolution from marine vertebrates to eutherian mammals in the way that the sperm surface molecules interact with the oligosaccharide ligands of the envelope glycoproteins (Töpfer-Petersen et al. 2008). The porcine egg coat, the *zona pellucida* (ZP), is composed of three glycoprotein families, ZP1 (ZPA; 92 kDa), ZP3α (ZPB; 55 kDa) and ZP3β (ZPC; 55 kDa) named by a nomenclature based on apparent molecular weight (Hedrick and Wardrip 1986, 1987). ZP1 is split into two smaller molecules, ZP2 (69 kDa) and ZP4 (23 kDa), under reducing conditions (Hasegawa et al. 1994). Sperm bind ZP by means of membrane receptors which recognize carbohydrate moieties on ZP glycoproteins according to a well-defined sequential process, one of which is the primary binding (Serres et al. 2008). The sperm-binding activity in pigs has been mapped to the neutral tri- and tetra-antennary complex N-glycans of ZPB expressing nonreducing terminal β-galactosyl residues (Kudo et al. 1998; Yonezawa et al. 2005).

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Upon ejaculation, the sperm cell surface is coated with extracellular glycoproteins that form a protective layer and stabilize the sperm cell. Sperm surface-coating factors are believed to mask underlying proteins involved in (1) sperm-ZP binding and (2), especially in pigs, docking of the acrosome required for the initiation of the acrosome reaction (Gadella 2013). This step has a vital purpose in pigs and some other mammals, as reviewed in Suarez (2008). To be able to selectively recognize ZP, the sperm must undergo the capacitation process, during which sperm ZP-binding proteins are ordered in functional protein complexes that only emerge at the apical tip of the sperm head plasma membrane, the exclusive area involved in primary ZP binding (Boerke et al. 2008; Gadella 2008; Gadella et al. 2008). Capacitated porcine spermatozoa exhibit stable docking of the acrosome to the plasma membrane, preparing the sperm for the acrosome reaction (Tsai et al. 2010). The physiological execution of the acrosome reaction is a later event, and, just recently, it has become less clear where this event is initiated.

Characterization of the molecules that mediate primary recognition and adhesion to ZP still remains a difficult task. Several putative ZP receptors have been identified in pig, including spermadhesins AWN, AQN-1 and AQN-3 as well as P47 (lactadherin) and the short Fn-2 type protein pB1 (also DQH) and carbonyl reductase (Ensslin et al. 1995; Jonáková et al. 1998; Maňásková et al. 2000, 2007; van Gestel et al. 2007). These data support the current concept of a multiple receptor involved in primary binding, in which the contributing proteins may act sequentially or synergistically.

Over the last few decades, one of the central dogmas of the fertilization process in mammals has been that once capacitated, acrosome-intact sperm bind to the ZP and then undergo acrosomal exocytosis (Saling et al. 1979). Recent experiments suggest that sperm binding to ZP is not sufficient to induce acrosomal exocytosis, and instead of ZP-triggered acrosomal exocytosis, Baibakov et al. (2007) proposed a mechanosensory mechanism that involved (1) the binding of acrosome-intact sperm to the ZP surface, followed by (2) the loss of the acrosome as the sperm penetrate the ZP. Furthermore, Jin et al. (2011) made a groundbreaking observation that, in the mouse at least, instead of the ZP, the cumulus appears to be the physiological inducer of the acrosome reaction. This was also observed earlier in pigs (Mattioli et al. 1998). Other inducers of acrosome reaction are progesterone (Melendrez et al. 1994) as well as estrogens (Děd et al. 2010).,

Acrosomal exocytosis ensures the exposure and release of soluble and acrosomal matrix proteins. The actual model of the penetration process includes alternating cycles of (1) binding of the acrosome-reacted sperm to the ZP (secondary binding), (2) limited proteolysis of the matrix and (3) release of the sperm and penetration caused by the sperm forward motility (O'Rand et al. 1986). Acrosin was believed to be the main participant of this model; however, skepticism came from the observation that mice sperm null for proacrosin were able to penetrate ZP and to fertilize the egg (reviewed by Honda et al. 2002).

Membrane molecules, which are the main ZP-binding candidates, often represent minor components in total cellular extracts, and therefore sophisticated isolation approaches must be used. Approaches for selective isolation of the surface subproteome have therefore been developed, including nitrogen cavitation (Canvin and Buhr 1989; Flesch et al. 1998; Bongalhardo et al. 2002), isolation of proteins associated with detergent-resistant membranes (DRM) (Cross 2004; van Gestel et al. 2005 and Girouard et al. 2008), and affinity isolation of tagged proteins. A promising technique involving tagging the surface molecules with sulfo-NHS-SS-Biotin [Sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] was introduced by Zhao et al. (2004). This approach was successfully implemented for the identification of new potential ZP-binding candidates (Belleannee et al. 2011; Zigo et al. 2013). Numerous studies have characterized sperm membrane receptors responsible for the binding to ZP in various animals, reviewed in Tanphaichitr et al. (2007).

The purpose of this study was to develop an alternative tool for monitoring and identification of ZP-"binding" receptors. This tool comprises a panel of monoclonal antibodies raised against proteins from the sperm surface. The following goals were defined: (1) preparation of the panel of monoclonal antibodies and their testing on epididymal, ejaculated and capacitated sperm for the protein localization; (2) localization of the proteins recognized by the panel in selected reproductive tissues and fluids; (3) screening for co-incidence in binding of the proteins recognized by the panel with ZP glycoproteins; (4) use of the panel for immunoprecipitation of selected proteins that coincide in ZP binding; and (5) sequencing of the precipitated proteins.

Materials and methods

Collection of biological fluids, spermatozoa and tissues from boar reproductive organs

Boar ejaculates from 12 adult animals were obtained from the breeding station PROAGRO Nymburk (Nymburk, Czech Republic) and pooled to obtain a representative "sample". Ejaculates were centrifuged (400*g*, 20 min) to separate seminal plasma from spermatozoa. Spermatozoa were washed three times with phosphate-buffered saline (PBS) and centrifuged for 10 min at 400*g*. Washed sperm samples were used for protein extraction and immunofluorescence.

For the experiments, reproductive fluids and reproductive and non-reproductive organ tissues from five adult fertile boars were collected immediately post-mortem from the Breeding Institute of Animal Physiology and Genetics Liběchov, Academy of Sciences of the Czech Republic, v.v.i., Czech Republic. Boar epididymal fluid together with

epididymal spermatozoa were obtained from the epididymal duct by injection and extrusion of the fluid. Epididymal fluid with spermatozoa was centrifuged for 20 min at 600*g*. Spermatozoa were washed four times with PBS and then centrifuged for 15 min at 400*g*.

Boar seminal vesicle fluid was obtained by the following procedure. The seminal vesicles, separated from connective tissue, were cut away from the urethra and the secretions were collected by applying pressure. After centrifugation (3500*g*, 15 min, 4 °C), the supernatant was frozen and stored at −25 °C.

Tissues of boar urogenital tract (from the testes and prostate) and tissue obtained from non-reproductive organ (kidneys) were homogenized in Tris-buffered solution [pH 7.8, 30 mM Tris, 50 mM KCl, 1 % (v/v) Triton X-100] by homogenizer Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France) according to the manufacturer's protocol. Homogenates were centrifuged (20,000*g*, 4 °C) and supernatants were stored at −25 °C.

Sperm capacitation

Sperm capacitation was done as described in Zigo et al. (2011). Briefly, fresh boar ejaculates diluted in KORINAT I (14.3 mM sodium bicarbonate, 12.25 mM sodium citrate, 364 mM glucose, and 12.3 mM EDTA; pH 7.5) were centrifuged at 400*g*. The sperm pellet was washed with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) to remove dilutor components, layered on a 40–80 % (v/v) discontinuous Percoll gradient (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 200*g* for 45 min. After centrifugation, the 80 % (v/v) layer was diluted in ten times diluted Tyrode's buffer medium (TBM) (pH 7.7; 20 mM Tris, 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 11 mM glucose) (Sigma), 5 mM pyruvic acid (Sigma), 1 ampoule of gentamycin (80 mg/2 ml) (Lek Pharmaceuticals, Ljubljana, Slovenia), and the cells were washed again. The washed spermatozoa were resuspended in TBM, supplemented with 1 mg/ml of bovine serum albumin SL 5 grade (Serva, Heidelberg, Germany), and capacitated (4 h, 37 °C, 5 % (*v/v*) CO_2) (Berger and Horton 1988). Sperm samples after capacitation were used for protein extraction and immunofluorescence.

Sperm stages, before and after capacitation, were studied by indirect immunofluorescence with the use of monoclonal antibody against intra-acrosomal protein proacrosin/acrosin (Acr-2), as described previously by Děd et al. (2010). Non-capacitated, capacitated, and acrosome-reacted sperm were counted from the total of 200 random sperm in both ejaculated and in vitro capacitated sperm samples. In the ejaculated boar samples (before capacitation), the average count of non-capacitated sperm was 78 %, while in the samples after capacitation, the average count of capacitated sperm was 70 % (Fig. S1), which corresponded to the previous results in Děd et al. (2010).

Preparation of solubilized *zona pellucida*

Solubilized ZP was prepared as described in Zigo et al. (2013). Briefly, porcine ovaries were obtained from slaughtered adult sows from the slaughterhouse in Český Brod (Czech Republic). Oocytes were released from frozen porcine ovaries in a meat grinder with ice-cold saline (0.15 M NaCl) and the homogenate was sieved through nylon screens as described by Hedrick and Wardrip (1986). The oocytes were purified by centrifugation in a discontinuous Percoll gradient (Sigma) (Hokke et al. 1994), collected from the 0–10 % (v/v) interface, washed in distilled water, and gently homogenized using a small glass homogenizer. *Zona pellucida* particles were collected on a 40 μm screen and repeatedly washed with saline. Isolated *zonae pellucidae* were heat solubilized in 0.2 M $NaHCO_3$, pH 9 at 73 °C for 30 min, and centrifuged at 350*g* for 10 min. The supernatant was used for biotinylation.

Biotinylation of *zona pellucida* glycoproteins

ZP glycoproteins were biotinylated as described in Zigo et al. (2013). Briefly, solubilized *zona pellucida* was incubated with 0.4 % (w/v) N-hydroxysuccinimidobiotin (Sigma) in dimethylformamide (Sigma) for 30 min at room temperature (Jonáková et al. 1998). *Zona pellucida* glycoproteins were dialyzed in a Spectra/Por MWCO 6/8000 membrane (Spectrum Medical, Laguna Hills, CA, USA) against phosphate-buffered saline (PBS; 20 mM phosphate, 150 mM NaCl, pH 7.2) overnight and stored at -25 °C.

Isolation of proteins from the sperm, preparation of protein extracts from tissues and fluids

The isolation of proteins from the sperm surface was done as described in Zigo et al. (2013). Briefly, a Thermo Scientific Pierce Cell Surface Protein Isolation kit (Rockford, IL, USA) was used according to the manufacturer's protocol. In this method, mammalian cells were first labeled with EZ-Link Sulfo-NHS-SS-Biotin, a thiol-cleavable amine-reactive biotinylation reagent. Cells were subsequently lysed with a mild detergent, and labeled proteins were then isolated with Immobilized NeutrAvidin Gel (agarose beads). The bound proteins were released by incubation with an SDS-PAGE sample buffer (50 mM Tris buffer titrated by HCl to pH 6.8, 1 % (v/v) glycerol, 2 % (*w/v*) SDS (Sigma), 0.002 % (w/v) bromophenol blue) containing 50 mM DTT (Sigma). The release of the bound proteins was completed on a multiple NeutrAvidin Gel column to achieve satisfactory protein yields (this is an extension of the manufacturer's protocol). Samples were stored at −25 °C prior to use.

The whole sperm extracts from the ejaculated and capacitated sperm (50 μl of sperm suspensions) were mixed with an SDS-PAGE sample buffer containing 50 mM DTT (Sigma),

vortexed and left to boil for 5 min. Sperm suspensions were centrifuged at 20,000*g* for 2 min at 4 °C. Supernatants were stored at −25 °C.

Prior to use, protein extracts from the sperm surface, whole sperm extracts, extracts from reproductive and non-reproductive tissues and collected fluids (epididymal, seminal vesicle and seminal plasma) were refined with 2-D Clean-Up Kit (GE Healthcare, Piscataway, NJ, USA) and all refined proteins were resuspended in SDS-PAGE sample buffer containing 50 mM DTT (Sigma).

Preparation of the panel of monoclonal antibodies against proteins from the sperm surface

Prior to immunization, boar sperm surface protein extract was clarified by using Zeba desalting spin columns (Thermo Scientific) to remove excess detergent and DTT, and used for immunization of BALB/c mice (AnLab, Prague, Czech Republic). Three females were immunized subcutaneously with 50 μg of sperm surface protein extract each in complete Freund's adjuvant (Sigma) followed by three additional immunizations with antigen in incomplete Freund's adjuvant at 2-week intervals. After 3 weeks, the final boost injection was performed intraperitoneally with no adjuvant followed by myeloma Sp2/0 and spleen cell fusion 3 days later according to the basic procedure (Harlow and Lane 1988). Positive clones were selected by indirect immunofluorescence on boar sperm cells. Hybridoma cells producing antibodies recognizing the apical part of the sperm head were subcloned and frozen for further use.

Sodium dodecyl polyacrylamide electrophoresis (SDS-PAGE), Western blotting

Protein extracts from the sperm, reproductive and non-reproductive tissues and collected fluids (epididymal, seminal vesicle and seminal plasma) obtained in the SDS sample buffer were used for one-dimensional electrophoresis (1-DE), which is a method for separation and analysis of macromolecules, based on their size and charge. SDS-PAGE was carried out as previously described by Laemmli (1970). A 7–21 % (w/v) gradient gel slab was used and run in a MiniProtean IV apparatus (Bio-Rad, Hercules, CA, USA). The concentration of bisacrylamide was 0.19–0.56 % (w/v), while the concentration of acrylamide was 6.81–20.44 % (w/v) from a total of 7–21 % (w/v). 15 μg of total protein was loaded per well. The molecular masses of the separated proteins were estimated by using prestained Precision Plus Protein Standards All Blue from Bio-Rad run in parallel.

Tris-glycine buffer (pH 9.6) with 20 % (v/v) methanol was used for the transfer of proteins separated by SDS-PAGE onto a PVDF Immobilon Transfer Membrane (Millipore, Bedford, MA, USA) for immunodetection. Electroblotting was carried out for 1.5 h at 500 mA, according to the method described by Towbin et al. (1979).

Protein immunodetection

The PVDF membrane (Millipore) with the transferred proteins was blocked with 1.5 % (w/v) teleostean fish gelatin (Sigma) in PBS for 3 h. After washing with 0.05 % (v/v) Tween 20 (Serva) in PBS, the membrane was incubated with primary antibodies from the panel of monoclonal antibodies against sperm surface proteins (diluted 1:50–1:200 in PBS) at 4 °C overnight. Following a washing step, incubation was performed for 1 h at 37 °C with goat anti-mouse immunoglobulins coupled to horseradish peroxidase (Sigma) diluted 1:12,000 in PBS. After washing, a chemiluminescent substrate, SuperSignal (Thermo Scientific), was applied and the blot was screened with ImageQuant LAS4000 (GE Healthcare) to visualize the corresponding interaction bands. Where not mentioned, procedures were carried out at room temperature. Blots were afterwards stained with Comassie Brilliant Blue (CBB; Serva).

Far-western blot with biotinylated *zona pellucida* glycoproteins

Far-western blot is derived from the standard Western blot method to detect protein–protein interactions in vitro. Far-western blot with biotinylated ZP glycoproteins was done as described in Zigo et al. (2013). Briefly, the PVDF (Millipore) membrane with the transferred proteins was deactivated with 1 % (w/v) teleostean fish gelatin (Sigma) in PBS for 4 h at room temperature. After washing with 0.02 % (v/v) Tween 20, 1 mM $CaCl_2$ in PBS (pH 7.2), the membrane was incubated with biotin-labeled glycoproteins of porcine *zona pellucida* (gpZP) (100 μg/ml in PBS) at 4 °C overnight. Following washing, incubation was performed for 0.5 h at 37 °C with 0.1 μg/ml of avidin-peroxidase solution (Sigma) in PBS. After washing, a chemiluminescent substrate, SuperSignal (Thermo Scientific), was applied and the blot was screened with ImageQuant LAS4000 (GE Healthcare) to visualize the corresponding interaction bands. Afterwards blots were stained with CBB (Serva).

The origin of the sperm surface subproteome was checked with the Western blot detection of acrosin and, within the limit of detection of this method, acrosin was present only in the avidine non-bound fraction after incubation of biotinylated sperm extract with avidine agarose beads. These results have been previously published in Zigo et al. (2013).

Immunoprecipitation

Protein extract (500 μg), dissolved in modified RIPA buffer (pH 7.2, 10 mM sodium phosphate, 150 mM NaCl,

1 % (v/v) Triton X-100, 1 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 1 mM DTT) (Sigma) with proteinase inhibitor cocktail Complete Mini (Roche, Mannheim, Germany), from capacitated boar sperm was incubated with monoclonal antibodies against sperm surface proteins (from the panel) in RPMI-1640 medium (Sigma) in a 7:4 volume ratio (700:400 μl) overnight at 4 °C. Then, 50 μl of Protein G-Sepharose beads (GE Healthcare) was added and incubated for 4 h at 4 °C. After centrifugation at 1000*g* for 1 min, protein G beads were washed six times with 200 μl PBS with 0.05 % (v/v) Tween 20 (Serva) and centrifuged at 1000*g* for 1 min. SDS-PAGE sample buffer containing 50 mM DTT (Sigma) was then added and the beads were boiled for 5 min and then centrifuged at 5000*g* for 10 min.

Supernatants were subjected to SDS-electrophoresis and the corresponding protein bands were detected with specific antibody on PVDF membranes. Immunoprecipitated proteins were subjected to mass spectrometric analysis.

Proteolytic digestion, sample preparation, and mass spectrometric analysis

The protein spots destaining, cysteine residue modification, proteolytic digestion, peptide extraction and sample preparation for mass spectrometry was performed as described previously (Sulc et al. 2009). The protein digestion was carried out in a cleavage buffer containing 0.05 M 4-ethylmorpholine acetate, 10 % (v/v) acetonitrile and sequencing grade trypsine endoprotease (Promega, 50 ng/μl) overnight at 37 °C. The resulting peptides were extracted with 40 % (v/v) acetonitrile/0.1 % (v/v) trifluoroacetic acid (TFA). After extraction, the peptides were directly diluted in 0.1 % (v/v) TFA and subjected to an R3 microcolumn (Applied Bioscience, Foster City, CA, USA) pre-equilibrated with 0.1 % (v/v) TFA. After desalting with the 0.1 % (v/v) TFA solution, the peptides were directly eluted with a 5 mg/ml solution of α-cyano-4-hydroxycinnamic acid in 50 % (v/v) acetonitrile/0.1 % (v/v) TFA from the R3 microcolumn on the MALDI target; and the droplets were allowed to dry at ambient temperature.

Mass spectra were measured in an ultraFLEX III matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-TOF/TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of peptide standards PepMix I (Bruker). The positive MALDI-TOF spectra and MS/MS LIFT spectra of the selected *m/z* signals were collected in reflectron mode to identify the proteins. MALDI-TOF MS and MS/MS spectra were interpreted using the MASCOT software engine (http://www.matrixscience.com/).

Indirect immunofluorescence technique: localization of the proteins on the surface of epididymal, ejaculated and capacitated sperm

Indirect immunofluorescence for localization of proteins of interest recognized with the panel of monoclonal antibodies on the surface (non-permeabilized membrane) of epididymal, ejaculated and capacitated sperm was used to assess the presence/absence and potential redistribution of these proteins during their individual states. Sperm suspensions were smeared onto glass slides and left to desiccate at ambient temperature. Primary antibodies from the panel were applied. Slides were left to incubate for 1 h at 37 °C in a moist chamber. After washing, incubation was carried out with a secondary antibody against the mouse IgG Fc fragment, conjugated with fluorescein isothiocyanate (FITC; Sigma) diluted 1:160 in PBS, for 1 h at 37 °C. Finally, after washing with PBS and distilled water, slides were incubated for 12 min with 1.5 μg/ml of VectaShield-DAPI (Vector Laboratories, Burlingame, CA, USA). Samples were viewed and evaluated with a Nikon Eclipse E400 fluorescent microscope with a ×100 Nikon Plan Fluor lens and a VDS CCD-1300 camera (VDS Vosskuhler, Osnabruck, Germany) with the aid of LUCIA imaging software (Laboratory Imaging, Prague, Czech Republic). In the controls, a non-sense primary antibody with a matched immunoglobulin subclass at the same concentration as the test antibody was used, and the procedure was followed as previously described; no reactions were observed (data not shown).

Additionally, a control to validate surface labeling by the antibodies to a known internal antigen, β tubulin, and phosphotyrosine, was performed to exclude the possibility that air-dried sperm smears could have cracked or perforated membranes so that antibodies could gain access to internal proteins. This control study is included in the Electronic supplementary material.

Results and discussion

Panel of monoclonal antibodies against proteins from the sperm surface

A panel of monoclonal antibodies against sperm surface proteins was prepared. The reason we decided to operate with the surface subproteome and not the whole sperm proteome was to increase our chances in obtaining antibodies against surface proteins, as these proteins often represent minor components in total cellular extracts. The panel comprised the 17 antibodies listed in Table 1, which also summarizes the experiments performed. Using the indirect immunofluorescence technique, of the total number of 17 antibodies 2 did not recognize and 4 recognized parts of the sperm tail, and the rest of the

Table 1 Characterization of panel of monoclonal antibodies

Ab	Stained region in ejaculated sperm	WB of sperm proteome	Immunofluorescence intensities of the sperm[a]			Ab recognized protein localization
			Epididymal	Ejaculated	Capacitated	
3B10	Midpiece	−	−	+	+	Not tested
1C6	Non-recognizing	−	−	−	−	Not tested
4C7	Acrosomal region	45 kDa	−	++++	+++	Surface of ejaculated and capacitated sperm
4C11	Acrosomal region	45 kDa	−	++++	+++	Surface of ejaculated and capacitated sperm
5C5	Acrosomal region	24, 27 kDa	+	++	++++	Surface of capacitated sperm, epididymal fluid
1D1	Acrosomal region	51 kDa	−	+	++++	Surface of capacitated sperm
2D10	Acrosomal region	200 kDa	−	++	+++	Surface of capacitated sperm
3D5	Principal piece	−	+	++	++	Not tested
3D7	Acrosomal region	−	−	+	+	No reaction
1E3	Acrosomal region	32, 35, 38 kDa	−	++	+++	Surface of ejaculated and capacitated sperm, epididymal fluid, prostate
2E1	Acrosomal region	200 kDa	+	++	+++	Surface of ejaculated and capacitated sperm
4E8	Midpiece	−	++	++	+++	Not tested
5 F2	Acrosomal region	−	+++	+++	++++	No reaction
1G7	Midpiece	−	+	++	++	Not tested
2G9	Non-recognizing	−	−	−	−	Not tested
1H9	Apical region	35, 45 kDa	−	++++	+++	Surface of capacitated sperm, kidney
2H10	Postacrosomal region	70 kDa	+	++	++	Not tested

[a] Immunofluorescence intensities: −no, + weak, ++ medium, +++ good, ++++ strong

antibodies stained the apical region of the sperm head (Table 1), where the primary receptors for ZP binding are located (Boerke et al. 2008). Therefore, the six above-mentioned antibodies were excluded from the panel. The next question was whether the remaining 11 antibodies were robust enough to also detect the protein of interest by Western blotting. Table 1 clearly shows that out of 11 antibodies recognizing the acrosomal part of the sperm head by indirect immunofluorescence, 8 also detected the protein of interest by Western blot (corresponding molecular masses of proteins recognized by the remaining 8 antibodies are listed in Table 1). These 8 monoclonal antibodies recognizing the acrosomal part of the sperm head and detecting the proteins of interest by Western blot were further studied and are discussed in the following text and the Electronic supplementary material.

Recently, the model of primary binding caused by the induction of acrosome reaction has become disputed, as it was found in mice that the majority of the sperm reaching ZP are already acrosomally reacted, and the acrosomal exocytosis is probably induced by cumulus (Jin et al. 2011). However, without knowing the exact locations where acrosomal exocytosis occurs during the course of normal fertilization, a role of the ZP in stimulating or inducing this sperm secretory event cannot be excluded. It may be that, during the biogenesis of the ZP within the ovarian follicle, ZP proteins diffuse into the extracellular of the cumulus cells surrounding the oocyte, either by not being incorporated into the particulate zona during assembly or by the slight degradation of ZP proteins after insertion into the zona. Further studies are required to investigate this possibility.

Antibody recognizing the protein of 45 kDa: 4C7

The monoclonal antibody from the panel termed 4C7, recognizing the protein of molecular mass ~45 kDa, was further studied. Using indirect immunofluorescence, the protein recognized by the 4C7 antibody was localized on the surface of non-permeabilized sperm. This protein was present both on ejaculated (Fig. 1a') and capacitated sperm (Fig. 1a"), but not on the surface of epididymal sperm (Fig. 1a). The signal was relatively strong on both ejaculated and capacitated sperm. Additionally, we searched for the origin of this protein in reproductive tissues and fluids. The result is depicted in Fig. 1b, clearly showing that the protein recognized by the 4C7 antibody was present solely on the surface of both ejaculated and capacitated sperm (Fig. 1b,– lanes 1), but in no other location. The whole sperm proteome extracts (Fig. 1b, lane 2) and extract from the kidney (Fig. 1b, lane 8) served as positive and negative controls, respectively. The molecular mass shifts of the proteins isolated from the sperm surface towards higher masses with respect to proteins isolated from the whole sperms are due to biotinylation. Next, we studied whether the protein recognized by the 4C7 antibody could bind ZP glycoproteins. We employed far-western blot assay of

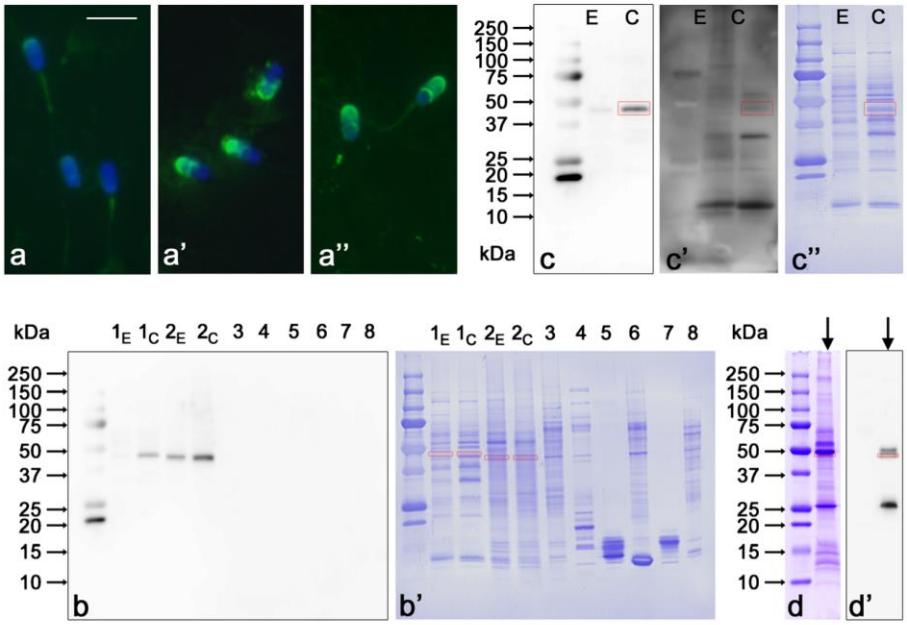

Fig. 1 Characterization of antibody termed 4C7 by immunofluorescence of epididymal (**a**), ejaculated (**a'**) and capacitated (**a"**) sperm; immunolocalization by Western blotting (**b**) in the sperm surface subproteome (*lanes 1*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, SDS extract (*lanes 2*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, extract from the testis (*lane 3*), epididymal fluid (*lane 4*), seminal vesicle fluid (*lane 5*), extract from the prostate (*lane 6*), seminal plasma (*lane 7*), and extract from the kidney (*lane 8*) followed by CBB staining (**b'**); comparison of Western blot detection (**c**) with the far-western blot using biotinylated *zona pellucida* glycoproteins (**c'**) of the sperm surface subproteome from ejaculated (*lane E*) and capacitated (*lane C*) sperm, respectively, followed by CBB staining (**c"**); and immunoprecipitation from capacitated sperm extract (**d**) confirmed by the Western blot (**d'**). All corresponding bands are indicated by *red rectangles*. The first lane in (**b**, **b'**, **c**, **c'**, **c"** and **d**, **d'**) represents molecular mass standards. *Bar* in immunofluorescence 10 μm

proteins isolated from the sperm surface with ZP glycoproteins (Fig. 1c') and the interactions were compared with Western blot detections (Fig. 1c). The results depicted clearly show that the protein recognized by 4C7 antibody coincides in binding to the *zona pellucida* at the molecular mass of 49 kDa. The interaction was more apparent with capacitated sperm (Fig. 1c', lane C) than with ejaculated sperm (Fig. 1c', lane E), which can be attributed to greater abundance of the protein in the capacitated fraction. Prior to mass spectrometry analysis, the protein was immunoprecipitated for higher purity and concentration (Fig. 1d) and the immunoprecipitate was tested by Western blot (Fig. 1d'). After SDS-PAGE, the protein band was located just under the heavy chain (50 kDa) of the 4C7 antibody, which was further confirmed by Western blotting (Fig. 1d, d'), and the third signal belonged to the light chain (25 kDa) of the 4C7 antibody. The protein of interest was identified after in-gel proteolysis and following MS and MS/MS analysis of formed peptides using the Mascot Peptide Mass Fingerprint tool to be an acrosin precursor (EC 3.4.21.10) (gi|164703 from *Sus scrofa*, with MW 46 kDa, pI 9.66), significant probability Mowse score 164 [protein scores greater than 72 are significant ($p<0.05$), sequence coverage 34 %, matched 10 from 13 searched *m/z* values]. The acrosin precursor identification was confirmed using MS/MS Ion Search of acquired MS/MS spectra at *m/z* 2578.389 [probability Mowse score 96, scores greater than 34 are significant ($p<0.05$), mass error 7.0 ppm, identified sequence R.LIFGANEVVWGSNKPVKPPLQER.F] and *m/z* 2139.089 [probability Mowse score 98; scores greater than 40 are significant ($p<0.05$), mass error 3.5 ppm, identified sequence K.RPGVYTSTWPYLNWIASK.I], respectively.

The identified protein is a major component of the acrosomal content, localized both in the inner acrosomal membrane and acrosomal matrix, which also plays a role in reproduction as a secondary binding receptor to the ZP (Tesařík et al. 1988; Jones and Williams 1990; Töpfer-Petersen and Calvete 1995, 1996). It has been shown in mice that knockout of the acrosin gene does not affect fertility (Baba et al. 1994), although it may provide a competitive advantage to wild-type relative to acrosin-null mouse sperm by promoting dispersion of the acrosomal matrix (Adham et al. 1997; Yamagata et al. 1998). In domestic animals such as the pig, which have oocytes surrounded by a thick ZP (16–20 μm), acrosin seems to essentially contribute to the secondary binding interaction and sperm penetration through the ZP (Töpfer-Petersen et al. 2008). This is not the first time we have detected acrosin on the surface of ejaculated and capacitated sperm (Zigo et al. 2013). The explanation may be that, in both ejaculated and

capacitated sperm samples, acrosomally reacted sperm were also present (Supplemental Fig. 1) and the released acrosin remained adherent to the sperm surface regardless of washing (Straus et al. 1981; Straus and Polakoski 1982). At this point, we believe that the presence of proacrosin/acrosin on the surface of the sperm, preferentially capacitated (Fig. 1b), is not coincidental, and that the spontaneous (false) acrosome reaction also has its meaning in the process of fertilization. We suggest that a portion of sperm which undergo spontaneous acrosome reaction also have a physiological function that may allow the released proacrosin/acrosin adhere to other acrosomally non-reacted sperm. The ability of acrosin to bind ZP is not a new attribute. Acrosin has been shown to participate in the secondary binding of the sperm to oocyte in multiple animal models (with acrosin localization on the inner acrosomal membrane). This is why the presence of acrosin on the surface of sperm is also of interest. Acrosin will certainly also bind ZP on the sperm surface, but the purpose of this will be different from the secondary binding of the sperm to oocyte. In this case, we presume that acrosin would most probably participate as a mediating molecule or even as a primary binding molecule. However, further experiments are required to confirm this hypothesis.

Antibody recognizing the protein of 24 and 27 kDa: 5C5

The monoclonal antibody termed 5C5 from the panel recognizing the protein of molecular masses ~24 and 27 kDa was further studied. Using immunofluorescence, the protein recognized by the 5C5 antibody was localized on the surface of non-permeabilized sperm. The protein was present on the surface of all epididymal, ejaculated, and capacitated sperm. The detected signal was strong in case of capacitated sperm (Fig. 2a"), while in epididymal (Fig. 2a) and ejaculated sperm (Fig. 2a') it was of medium strength. Additionally, we searched for the origin of this protein in reproductive tissues and fluids. The result is depicted in Fig. 2b, showing clearly that the protein is present only on capacitated sperm, with only the 24-kDa form present, with no signal on ejaculated sperm (Fig. 2b, lanes 1). The protein originates from epididymal fluid, where both 24- and 27-kDa forms are present (Fig. 2b, lane 4). The whole sperm proteome extracts (Fig. 2b, lanes 2) and extract from the kidney (Fig. 2b, lane 8) served as positive and negative controls, respectively. Extraction of proteins from ejaculated and capacitated sperm with SDS yielded both 24- and 27-kDa forms of the protein (Fig. 2b, lanes 2). The extraction conditions during isolation from the sperm surface allowed us to obtain only the 24-kDa form from the surface of capacitated sperm (Fig. 2b, lanes 1). Overall, this suggests that during the passage via epididymis the protein recognized by the 5C5 antibody is integrated from the epididymal fluid into the surface of the sperm. Further, we were unable to detect the protein by Western blot during ejaculation, when the proteins from seminal plasma are bound to the sperm surface and make the protein of interest inaccessible to biotinylation. During capacitation, when the bound proteins are released from the sperm surface, the protein was biotinylated and observed. However, the fact that only the 24-kDa form was obtained by the method for isolating proteins from the sperm surface indicates that the 27-kDa form is firmly anchored in the sperm plasma membrane.

We next investigated whether the protein recognized by the 5C5 antibody could bind ZP glycoproteins. As previously, we also used the far-western blot with ZP glycoproteins (Fig. 2c'), but with the whole sperm proteome because the protein was more abundant in these extracts, as evidenced by Fig. 2b. The interactions were compared with Western blot detections (Fig. 2c). The results show that the protein recognized by 5C5 antibody coincides in binding to *zona pellucida* of capacitated sperm (lane C) at the molecular mass of 24 kDa. The interaction is more apparent with capacitated sperm (Fig. 2c', lane C) than with ejaculated sperm (Fig. 2c', lane E), which again can be attributed to greater abundance of the protein in the capacitated fraction (Fig. 2b). We assume that the 27-kDa form also coincides in binding to *zona pellucida*, but the direct statement based on Fig 2c' is rather speculative. We were not able to clarify this even after multiple far-western blot assays, and we therefore concentrated only on the 24-kDa form. Prior to mass spectrometry analysis, the protein was immunoprecipitated for higher purity and concentration (Fig. 2d) and the immunoprecipitate was tested by Western blot (Fig. 2d'). After SDS-PAGE, the protein band was located under the light chain of the 5C5 antibody, which was further confirmed by Western blotting (Fig. 2d, d'), and the third signal of 50 kDa belonged to the heavy chain of the 5C5 antibody. The protein of interest was identified after in-gel proteolysis and following MS and MS/MS analysis of formed mixture of peptides using the Mascot Peptide Mass Fingerprint tool to be a Ras-related protein RAB-2A (gi|311253799 from *Sus scrofa*, with MW 24 kDa, pI 6.08), significant probability Mowse score 91 [protein scores greater than 76 are significant ($p<0.05$), sequence coverage 31 %, matched 5 from 7 searched *m/z* values]. The protein identification was confirmed using MS/MS Ion Search of acquired MS/MS spectra at *m/z* 1550.739 [probability Mowse score 103, scores greater than 45 are significant ($p<0.05$), mass error 12.8 ppm, identified sequence K.LQIWDTAGQESFR.S] and *m/z* 1785.894 [probability Mowse score 118, scores greater than 45 are significant ($p<0.05$), mass error 3.52 ppm, identified sequence R.FQPVHDLTIGVEFGAR.M], respectively.

RAB proteins belong to a subgroup of the Ras superfamily, whose four members were first identified in the brain (Touchot et al. 1987). Presently, the RAB family includes over 60 members in the human genome (Bock et al. 2001), thus being the largest branch of the Ras-related family of low-molecular-weight GTP-binding proteins. RAB proteins have been shown

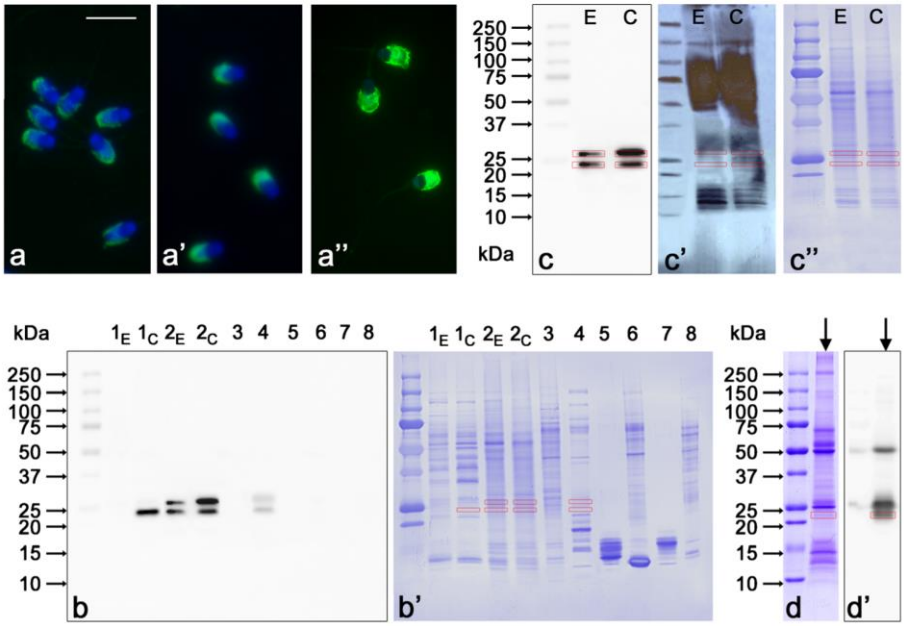

Fig. 2 Characterization of antibody termed 5C5 by immunofluorescence of epididymal (**a**), ejaculated (**a'**) and capacitated (**a"**) sperm; immunolocalization by Western blotting (**b**) in the sperm surface subproteome (*lanes 1*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, SDS extract (*lanes 2*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, extract from the testis (*lane 3*), epididymal fluid (*lane 4*), seminal vesicle fluid (*lane 5*), extract from the prostate (*lane 6*), seminal plasma (*lane 7*), and extract from the kidney (*lane 8*) followed by CBB staining (**b'**); comparison of Western blot detection (**c**) with the far-western blot using biotinylated *zona pellucida* glycoproteins (**c'**) of the sperm surface subproteome from ejaculated (*lane E*) and capacitated (*lane C*) sperm, respectively, followed by CBB staining (**c"**); and immunoprecipitation from capacitated sperm extract (**d**) confirmed by the Western blot (**d'**). All corresponding bands are indicated by *red rectangles*. The first lane in (**b**, **b'**, **c**, **c'**, **c"** and **d**, **d'**) represents molecular mass standards. *Bar* in immunofluorescence 10 μm

to play an essential role as regulators of vesicular transport pathways (Pereira-Leal and Seabra 2000, 2001). They are involved in many stages of vesicular transport including vesicle formation, actin- and tubulin-dependent vesicle movement, and targeting to and fusion with membranes (Stenmark and Olkkonen 2001), enabling them to accomplish a diverse set of functions by interacting with a multitude of effectors. When RABs are first produced, they are prenylated by the addition of one or two 20-carbon geranylgeranyl moieties to the protein's carboxyl terminus (Stenmark and Olkkonen 2001), which are used to anchor the RABs into membranes. Multiple targeting determining regions and factors contribute to the specificity and regulation of RAB recruitment and localization (Ali and Seabra 2005). RAB2 proteins are typically found between the cis-Golgi saccule and the endoplasmic reticulum (Stenmark and Olkkonen 2001). In this cytosolic location, they are normally involved in orchestrating both anterograde and retrograde transport between these two membrane compartments (Short et al. 2001; Cheung et al. 2002). However, recently, it was found that RAB-2A may also participate in events localized within the germ cell. Mountjoy et al. (2008) demonstrated that RAB-2A protein is involved in acrosomal biogenesis, where it regulates the transport and fusion of small secretory vesicles to the growing proacrosomic and acrosomic vesicles and ensures their fusion. After completion of acrosomal biogenesis, RAB-2A serves as a part of the perinuclear theca protein complex that binds the acrosome firmly to the nucleus, thus stabilizing the acrosome. Additionally, Mountjoy et al. (2008) also showed the difference in orientation or cellular polarity of the Golgi apparatus in the spermatid versus the somatic cell.

We found the RAB-2A protein on the surface of boar sperm. To our knowledge, we were the first to detect its presence in the pig germ cell. In contrast to Mountjoy et al. (2008), we detected the RAB-2A protein on the surface of the sperm plasma membrane. Immunofluorescent microscopy study revealed that RAB-2A was localized on the surface of all epididymal, ejaculated and capacitated sperm. Extraction of the whole sperm proteome showed that two forms of RAB-2A are present in the pig sperm, of 24 and 27 kDa. The presence of more than one RAB-2A form may be explained by hypervariability of the C-terminal domain, as shown in Chavrier et al. (1991). However, only the 24-kDa form was obtained from the surface subproteome, and from capacitated sperm only. We believe that the 27-kDa form, in contrast to the 24-kDa form, possesses additional geranylgeranyl moieties, as previously shown in RAB proteins (Stenmark and Olkkonen 2001), and therefore is more resistant to isolation

under mild conditions. As previously described, RAB-2A originates from the epididymal fluid. Considering that RAB proteins are commonly prenylated to be anchored in the membranes, the most probable way how RAB-2A is secreted into the epididymal lumen is through the membranous secretory vesicles—exosomes called epididymosomes, reviewed in Simpson et al. (2008). Epididymosomes are small membranous vesicles secreted in an apocrine manner in the intraluminal compartment of the epididymis, which also play a major role in the acquisition of new proteins by the maturing spermatozoa (Sullivan et al. 2007). Despite the association of RAB-2A protein with epididymosomes not yet having been proved, Girouard et al. (2011) found other RAB family proteins to be associated with these membranous vesicles in bull. Furthermore, Utleg et al. (2003) have shown that RAB-2 proteins in humans are associated with exosomes originating from the prostate, so-called prostasomes. The function of RAB-2A on the sperm surface still remains unsolved. According to Integrative Multi-species Prediction (Wong et al. 2012), there is 35 % probability that RAB-2A has a role in reproduction, as it was predicted that RAB-2A participates in gamete generation (Mountjoy et al. 2008) with the same probability. We have shown that RAB-2A, at least its 24-kDa form, coincides in binding to ZP. However, if RAB-2A can bind the ZP, it should be further clarified whether it binds as a primary ZP receptor or a primary binding-mediating molecule.

Antibody recognizing the protein of 35 and 45 kDa: 1H9

The monoclonal antibody termed 1H9 from the panel recognizing the protein of molecular mass ~35 and 45 kDa was further studied. Using indirect immunofluorescence, the protein recognized by the 1H9 antibody was localized on the surface of non-permeabilized sperm. The protein was present both on ejaculated (Fig. 3a') and capacitated sperm (Fig. 3a"), but the signal on the surface of epididymal sperm was absent (Fig. 3a). The signal was relatively strong both on ejaculated and capacitated sperm, and was shifted from the apical region of the ejaculated sperm to the postacrosomal region. We also searched for the origin of the protein recognized by the 1H9 antibody in the reproductive tissues and fluids. The result is depicted in Fig. 3b, which clearly shows that the protein is present on the surface of capacitated sperm (Fig. 3b, lanes 1), and its faint signal was detected on the surface of ejaculated sperm and also in the kidney. The whole sperm proteome extracts (Fig. 3b, lanes 2) and extract from the kidney (Fig. 3b, lane 8) served as positive and negative controls, respectively.

We next investigated whether the protein could bind ZP glycoproteins. Similarly, as in the case of 4C7 and 5C5 antibodies, we employed far-western blot assay of proteins isolated from the sperm surface with ZP glycoproteins (Fig. 3c'), and the interactions were compared with Western blot detections (Fig. 3c). The results clearly show that the protein recognized by the 1H9 antibody coincides in binding to *zona pellucida* at the molecular masses of both 35 and 45 kDa. The interaction is more apparent with capacitated sperm (Fig. 3c', lane C) rather than with ejaculated sperm (Fig. 3c', lane E), probably due to greater abundance of the protein isolated in the capacitated fraction. Prior to mass spectrometry analysis, the protein was immunoprecipitated for higher purity and concentration (Fig. 3d) and the immunoprecipitate was tested by Western blot (Fig. 3d'). After SDS-PAGE, the protein band was located between the heavy and light chains of the 1H9 antibody, which was further confirmed by Western blotting (Fig. 3d, d'). Both bands of the interesting protein were identified after in-gel trypsinization and following MS and MS/MS analysis of formed peptides using the Mascot Peptide Mass Fingerprint tool to be a sperm surface protein SP47 (gi|2851513 from *Sus scrofa*, with MW 46 kDa, pI 6.15). The protein band at the molecular weight 45 kDa revealed significant probability Mowse score 235 [protein scores greater than 76 are significant ($p<0.05$), sequence coverage 41 %, matched 14 from 16 searched *m/z* values]. The protein identification was confirmed in this protein band using MS/MS Ion Search of acquired MS/MS spectra at *m/z* 1704.922 [probability Mowse score 119, scores greater than 45 are significant ($p<0.05$), mass error 7.0 ppm, identified sequence K.VNLFEVPLEVQYVR.L] and *m/z* 1851.868 [probability Mowse score 101, scores greater than 43 are significant ($p<0.05$), mass error 8.3 ppm, identified sequence R.TWGLSAFSWYPFYAR.L], respectively. Similarly, the significant probability Mowse score 224 [protein scores greater than 76 are significant ($p<0.05$), sequence coverage 34 %, matched 12 from 12 searched *m/z* values] was obtained for the protein band at the molecular weight 35 kDa and for the verification of protein identification the MS/MS Ion Search of acquired MS/MS spectra was performed. The MS/MS signal at *m/z* 1537.728 revealed probability Mowse score 83 [scores greater than 44 are significant ($p<0.05$), mass error 6.0 ppm, identified sequence R.AGIVNAWTASNYDR.N] and *m/z* 1704.92 [corresponding probability Mowse score 100, scores greater than 45 are significant ($p<0.05$), mass error 7.0 ppm, identified sequence K.VNLFEVPLEVQYVR.L], respectively.

The identified protein was previously described by Ensslin et al. (1998) as a novel peripherally associated 47-kDa protein of pig spermatozoa, P47, isolated by affinity chromatography from solubilized sperm plasma membrane proteins bound to immobilized *zona pellucida* glycoproteins. The pig sperm protein is homologous to lactadherins, major components of the milk fat globule membrane of the mammary gland (formerly known as bovine PAS 6/7 or MGP 53/57 and mouse MFG-E8; Larocca et al. 1991; Aoki et al. 1995; for review and recommended nomenclature, see Mather 2000). We were able to detect the P47/SP47 protein by immunofluorescence microscopy on the apical ridge of ejaculated sperm, which is in agreement with the results of Ensslin et al. (1998) and Petrunkina et al. (2003). Further, Petrunkina et al. (2003) showed that, after

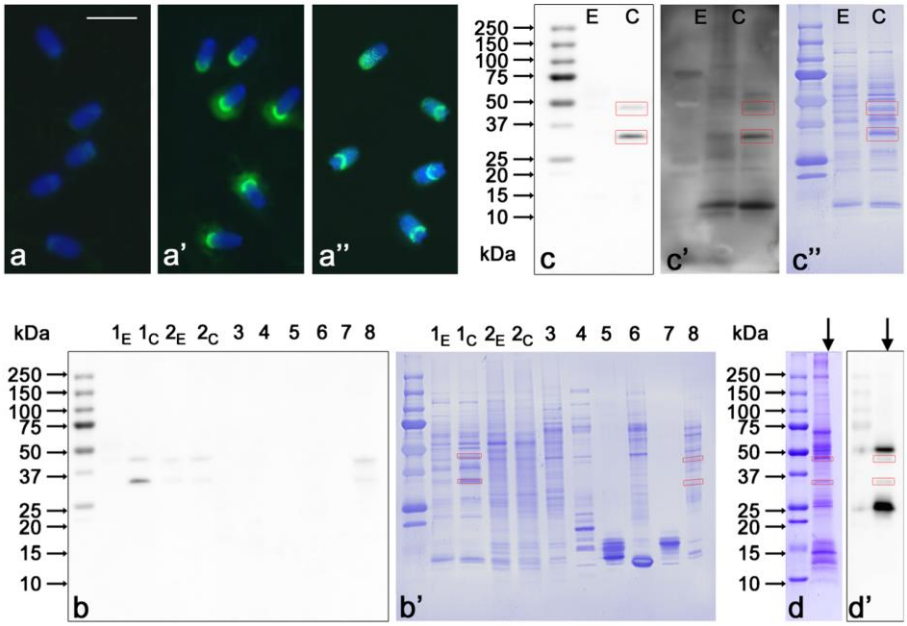

Fig. 3 Characterization of antibody termed 1H9 by immunofluorescence of epididymal (*a*), ejaculated (**a'**) and capacitated (**a"**) sperm; immunolocalization by Western blotting (**b**) in the sperm surface subproteome (*lanes 1*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, SDS extract (*lanes 2*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, extract from the testis (*lane 3*), epididymal fluid (*lane 4*), seminal vesicle fluid (*lane 5*), extract from the prostate (*lane 6*), seminal plasma (*lane 7*), and extract from the kidney (*lane 8*) followed by CBB staining (*b'*); comparison of Western blot detection (*c*) with the far-western blot using biotinylated *zona pellucida* glycoproteins (*c'*) of the sperm surface subproteome from ejaculated (*lane E*) and capacitated (*lane C*) sperm, respectively, followed by CBB staining (**c"**); and immunoprecipitation from capacitated sperm extract (**d**) confirmed by the Western blot (**d'**). All corresponding bands are indicated by *red rectangles*. The first lane in (**b**, **b'**, **c**, **c'**, **c"** and **d**, **d'**) represents molecular mass standards. *Bar* in immunofluorescence 10 μm

capacitation, the P47 signal shifted to the entire acrosomal distribution. Although in capacitated sperm we detected a small subpopulation of sperm having P47/SP47 unmasked, with resulting acrosomal appearance of the protein, the majority of the sperm displayed the fluorescence signal shifted to the postacrosomal region. In contrast to Ensslin et al. (1998), we were unable to obtain adequate fluorescent signals from epididymal sperm, most probably due to the inaccessibility of the epitope, as sperm were neither fixed nor permeabilized. We attempted to locate P47/SP47 in the reproductive organs and fluids, but due to relatively weak binding of the prepared antibody to the epitope, we were able to obtain a clear signal only with the capacitated sperm surface subproteome, as the isolation protocol for sperm surface proteins was modified to obtain enriched yields. In the ejaculated surface subproteome, only a faint signal was observed, which agrees with the results from immunofluorescence showing that only the protein located in the apical ridge was accessible for biotinylation. We also localized the presence of P47/SP47 in the kidney, in accordance with Ensslin et al. (1998), who observed that the protein is also expressed in non-reproductive organs such as muscle, heart, kidney, etc. Moreover, Ensslin et al. (1998) also showed that P47/SP47 is expressed in the following reproductive organs: uterus, cauda, corpus, caput epididymis, and testes. Furthermore, we detected two forms of P47/SP47: higher molecular mass form of 46 kDa (according to MASCOT software engine) and lower molar mass form of 35 kDa, not reported previously. Both forms were detected in ejaculated and capacitated sperm extracts. We believe that the 35-kDa form is either a truncated version of P47/SP47 or a processing product.

The function of lactadherin in association with spermatozoa still remains unclear. The possible function of boar membrane P47 as an integrin RGD-dependent ligand was suggested by Ensslin et al. (1998), previously indirectly supported by the studies of Andersen et al. (1997). Moreover, these investigators have recently shown that lactadherin can act as a link between two surfaces by binding to integrin receptors through its N-terminal RGD-binding sites in the second EGF-like domain and to phospholipids through its C-terminal C1/C2-like domains (Andersen et al. 2000). Taylor et al. (2000) demonstrated that human lactadherin (formerly BA46) expressed in human milk and breast carcinomas promotes RGD-dependent cell adhesion via integrins. Petrunkina et al. (2003) suggested that lactadherin is involved in other aspects of sperm physiology such as capacitation and acrosome reaction. However, further study is required to determine whether lactadherin epitopes are triggered or integrated in a further signal cascade priming the acrosome reaction and preparation of the sperm–egg fusion.

Fertilization in mammals is far from being completely understood, and recently new results have shown that one of the central dogmas of the fertilization process in mammals has become questioned. That is why it is necessary to continue with unrelenting efforts in order to move forward on the path of knowledge. This study dealt with an employment of monoclonal antibodies raised against the sperm surface proteins, localized on the apical tip of the sperm head plasma membrane, where the molecules for the ZP interaction are localized. We identified three proteins coincident with ZP binding: acrosin, RAB-2A and P47/SP47 lactadherin. Physiological function of P47 was proposed earlier. Due to the fact that acrosin stays adherent on the sperm plasma membrane, we propose an additional function, which is different from the secondary binding of sperm to oocyte. This is the first time to our knowledge that RAB-2A has been reported on the sperm surface, and the function of it remains undisclosed. The additional function of acrosin and the function of RAB-2A on the sperm surface are subjects for further studies.

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Conflict of interest The authors declare no conflicts of interest.

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Effect of zearalenone on reproductive parameters and expression of selected testicular genes in mice

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ABSTRACT

We tested the effect of two different concentrations (150 μg/l and 0.15 μg/l) of mycotoxin zearalenone (ZEA) on the reproductive parameters and expression of testicular genes in male mice. In adult males, no reduction of body or reproductive organ weight was observed, and the seminiferous tubules were morphologically normal with ongoing spermatogenesis. However, we found decreased sperm concentration, increase of morphologically abnormal spermatozoa and increased binding of apoptotic marker annexin V. This study was also focused on the evaluation of gene expression profiles of 28 genes playing important roles during the processes occurring in the testicular tissue. We detected changes in the expression of genes important for proper spermatogenesis. Surprisingly, we observed a stronger effect after exposure to the lower dose of ZEA.

1. Introduction

Zearalenone (ZEA, F-2 toxin) is a nonsteroidal oestrogenic mycotoxin produced by a variety of *Fusarium* fungi, which are common contaminants of cereal crops worldwide [1]. ZEA is commonly found in maize or corns with the highest concentration in wheat, bran, corn and their products (*e.g.* corn flakes). ZEA is mainly a field contaminant; however the toxin production can also occur during storage in poor conditions [2]. Also, it has been shown that ZEA is transported from the fields to the aquatic systems by rain water [3].

The concentrations in food and feed vary over a wide range, depending on climatic conditions. Considering the mean levels of ZEA in the principal foods and their consumption, the average human daily intakes of ZEA range from 2.4 to 29 ng/kg b.w./day in adults, while toddlers (12–36 months old) have the highest average daily intakes ranging from 9.3 to 100 ng/kg b.w./day [2]. It has been shown that ZEA can also be excreted into cow milk [4].

ZEA is rapidly absorbed after oral administration. Its uptake is estimated to be approximately 80–85%, but it is difficult to measure owing to extensive biliary excretion. ZEA and its derivatives are detected in blood about 30 min after oral administration bound to human globulins as reproductive hormones [5,6]. Studies with radiolabelled zearalenone in mice showed that it is distributed to oestrogen target tissues such as uterus, interstitial cells of the testes and ovarian follicles. Some radiolabels were also found in adipose tissues, indicating that storage in adipose tissue may take place [7].

The main effect of zearalenone results from its oestrogenic activity. ZEA and its derivatives – α-zearanenol (α-ZOL) and β-zearalenol (β-ZOL) – compete with 17β-estradiol (E2) for the specific binding sites of oestrogen receptors (ERs). Several investigations have demonstrated that binding of ZEA and its derivatives initiates a sequence of events known to follow oestrogen stimulation [7]. Efficiency of binding of ZEA to ER in target tissues is <1–10% than that of E2, whereas α-ZOL shows stronger binding and β-ZOL lower affinity to ER [8]. The specific manifestations of ZEA are dependent upon the species, relative dose, and life stage during which ZEA is consumed. The most sensitive species is the pig; however it has been shown that ZEA can also have adverse effects on other species including rodents.

A study by Yang et al. [9] has shown that ZEA and α-ZOL affect steroidogenesis in mature mouse Leyidig cells *in vitro*. During this study authors observed a decrease of testosterone production in cells co-treated with ZEA or α-ZOL and human chorionic gonadotropin (hCG). They also detected decreased

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expression of 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD-1), cytochrome P450 side chain cleavage enzyme (P450scc) and steroidogenic acute regulatory protein (StAR), which play a crucial role during steroidogenesis. In adult animals testosterone is critical for proper spermatogenesis and sperm maturation, and disruption of spermatogenesis can thus adversely affect male fertility.

The negative effect of ZEA on reproductive parameters can also be observed *in vivo*. In another study of Yang et al. [10], adult male mice were exposed to intraperitoneal injections of ZEA or α-ZOL at the concentration 0, 25, 50 or 75 mg/kg b.w. daily for 7 days. In all groups the authors observed a significantly increased number of abnormal spermatozoa and significantly decreased number of live spermatozoa. Testicular and cauda epididymal sperm counts were also reduced, as well as serum testosterone. These effects were observed in the treated males at all doses in a dose-dependent manner. Besides the decrease in sperm quality, a significantly low pregnancy rate was observed when untreated females were mated with the treated males. At high concentrations (50 and 75 mg/kg b.w.), authors noticed a decrease of b.w. and increase of relative seminal vesicle weight.

To show whether the action of ZEA includes induction of apoptosis of testicular cells, Kim et al. [11] performed an *in vivo* study in rats. During this study 10-week-old male rats were exposed to a single intraperitoneal dose of ZEA (5 mg/kg b.w.) and analyzed at 3, 6, 12, 24, or 48 h after exposure. Germ cell degeneration caused by apoptosis was observed at stages I–VI of spermatogenesis 12 h after the exposure. The frequency of TUNEL-labelled germ cells increased in a stage-specific manner, with gradually increasing frequency at stages I–VI of seminiferous tubules with the time after exposure. These results show that a single dose of ZEA induces testicular germ cell apoptosis in a time-dependent and stage-specific manner in the rat testis *in vivo*. The induction of apoptosis in testicular tissue after ZEA treatment was also shown by Yuan et al. [12]; these authors have additionally shown that traditional medicinal plant *Gynostemma pentaphyllum* protects against toxicity caused by ZEA through anti-oxidation and anti-apoptosis effects mediated by the regulation of Bax and Bcl-2 expression.

Filipiak et al. [13] performed an *in vivo* study of pubertal rats in which they investigated the effect of two xenoestrogens, diethylstilbestrol (DES) and ZEA, with comparison of their effect with natural oestrogen 17β-estradiol (E2). While E2 and DES significantly reduced the numbers of spermatogonia, spermatocytes and Sertoli cells, ZEA only reduced the numbers of spermatogonia and Sertoli cells. The authors also measured the testis weight and seminiferous tubule diameter and length, which were significantly decreased by all three substances. In general, ZEA appeared to be the weakest of the three oestrogenic substances.

The aim of the present study was to assess the effect of treatment with a low dose of mycotoxin zearalenone on the male gonadal pathology, sperm quality and expression of selected genes. We have analyzed a wide range of genes expressed in the testes. For this purpose we selected genes playing important roles during spermatogenesis, genes expressed in Sertoli cells, and genes playing a role in apoptosis and hormonal response. We assume that analysis of these genes can reveal how ZEA affects germ cell development and subsequently the semen quality in mice.

2. Materials and methods

2.1. Animals and treatment, number of progeny, sex ratio

For our experiment we used the CD1 outbred mice strain (An Lab, Prague, Czech Republic) with high heterozygosity and average litter size (12–13 pups/litter). Mice (experimental and control groups) were kept under standard experimental conditions (constant temperature and 12-h light regime) in the animal facility of the Institute of Molecular Genetics, v. v. i., Prague. Animals were fed on soy-free feed (Ssniff, Soest, Germany). The diet and water were administered *ad libitum* and all stress factors were reduced to a minimum. Experimental groups were treated with different concentrations of ZEA (Sigma, Prague, Czech Republic), which was dissolved in drinking water. In this *in vivo* experiment there were two experimental groups – a group exposed to higher concentration of ZEA (150 μg/l), and a second group exposed to lower concentration of ZEA (0.15 μg/l). Animals exposed to the low dose were exposed to an environmentally relevant concentration (around 25 ng/kg b.w.) and animals exposed to the high dose were exposed to 1000 times higher concentration. ZEA was administered starting from the first day of mothers' pregnancy, and the number of progeny and the sex ratio were evaluated. The born pups were exposed during gestation, lactation, pre-pubertal and pubertal period, and up to the age of 70 days, when they were sacrificed and subjected to analysis. In each group, 18 animals were analyzed.

2.2. Anogenital distance, body and organ weight

Animals in the control and experimental groups were killed at the same age of 70 days. Anogenital distance (AGD) and body weight were measured and subsequently the reproductive organs were dissected and weighed. Left and right testes, epididymis, seminal vesicles and prostate were separated, cleaned and weighed individually. Immediately after weighing the left testes were frozen in liquid nitrogen and stored at −70 °C for further analysis; the right testes were used for histological analysis and epidymides were placed into warmed (37 °C) PBS for sperm release (see below).

2.3. Preparation of cells

Mouse sperm were obtained from the cauda epididymis. Spermatozoa were left to release spontaneously into 1 ml of warmed PBS at 37 °C in a CO_2 incubator for 15 min. Then the cell suspension was transferred into a new tube and PBS was added to 1 ml final volume. The concentration of spermatozoa was evaluated in a haemocytometer chamber under 100× magnification. Part of the epididymal spermatozoa was used for assessing viability, morphology and apoptotic stage. The rest of the suspension was washed twice in PBS, centrifuged for 15 min at 200 × *g* and dropped onto glass slides for immunocytochemical analysis.

2.4. Sperm viability

To determine the viability of epididymal spermatozoa a Live/Dead sperm viability kit was used (Invitrogen, Eugene, USA); the laboratory manual had to be slightly modified to enable its use for mouse sperm. First, the cell suspension was centrifuged for 5 min at 200 × *g*, then 1 μl of cell pellet was mixed with 200 μl of HEPES buffer and 1 μl 50× diluted SYBR 14 (in HEPES), and the mixture was incubated for 5 min at 37 °C in the dark. Subsequently, the mixture was centrifuged for 5 min at 200 × *g*. The supernatant was removed and the cell pellet was mixed with 200 μl of HEPES buffer and 1 μl propidium iodide (PI) and centrifuged for 5 min at 200 × *g*. The supernatant was removed and 20 μl of PBS was added, then 10 μl of the suspension was placed onto a glass slide and evaluated under a Nikon Eclipse E400 fluorescence microscope using a 40× Nikon Plan Fluor 40/0.75 (Nikon, Prague, Czech Republic). Green (SYBR 14)-labelled spermatozoa were alive; orange-labelled (PI) spermatozoa were dead.

2.5. Sperm morphology

To evaluate the morphological state of spermatozoa, 10 μl of sperm cell suspension was placed onto a glass slide, fixed at 37 °C and labelled according to the protocol using Spermac Stain System (Ferti Pro, Beernem, Belgium). We evaluated at least 200 cells from each sample. Another method used to evaluate the sperm morphological status was indirect immunofluorescence with specific monoclonal antibody (Hs-14) against anti-acrosomal protein (see below).

2.6. Indirect immunofluorescence

Monoclonal antibody against intra-acrosomal protein (Hs-14) was used to assess the integrity of the acrosome. This antibody was prepared in our laboratory and is routinely used to test the acrosome state [14,15]. Epididymal spermatozoa were dropped on glass slides, air dried and fixed for 10 min with acetone. After rinsing with PBS, the slides were incubated overnight at 4 °C with monoclonal antibody (diluted to an immunoglobulin concentration 20 μg/ml). After thorough washing with PBS, the slides were incubated with anti-mouse IgM (μ-chain specific) fluorescein isothiocyanate (FITC) conjugate (Sigma, Prague, Czech Republic) diluted 1:128 in PBS and incubated for 60 min at 37 °C, washed with PBS and distilled water, and mounted in Vectashield H-1200 DAPI (Vector Laboratories, Burlingame, CA) for DNA visualization.

2.7. Apoptotic stage of spermatozoa

Apoptotic spermatozoa were detected using the APO-AF kit (Annexin V-FITC Apoptosis Detection Kit, Sigma, Prague, Czech Republic). The laboratory manual was slightly modified; 1 μl of cell suspension was mixed with 100 μl of 1× concentrated binding buffer and 1 μl of Annexin V solution. The mixture was incubated in the dark for 15 min at room temperature and centrifuged for 5 min at 200 × *g*. Supernatant was removed and 10 μl vectashield with DAPI was added. The mixture was placed onto a glass slide and examined with a Nikon Eclipse E400 fluorescent microscope equipped with a Nikon Plan Apo VC 60/1.40 oil objective and photographed with a CCD 1300-VDS camera (Vosskühler, Osnabrück, Germany) with the aid of the NIS-ELEMENTS AR imaging software (Laboratory Imaging, Prague, Czech Republic).

2.8. Histological analysis

The right testis was fixed in 4% formaldehyde in PBS for 48 h. Then the tissue was washed for 15 min in distilled water and dehydrated by increasing concentrations of ethanol. Subsequently, the tissue was placed in the mixture of acetone and xylene (1:1) for 30 min and then in xylene for 30 min. Finally, the testes were embedded in paraffin (Paraplast, Sigma, Prague, Czech Republic) and these paraffin blocks were cut in a microtome (sections 5 μm). For histological analysis the tissue sections were rehydrated by decreasing concentrations of ethanol and stained with haematoxylin-eosin. Tissue specimens were evaluated under a light microscope Olympus BX41 at 400× magnification and the images were photographed by camera Olympus IX81 (Olympus, Prague, Czech Republic).

2.9. TUNEL analysis

For detection of apoptotic cells in the paraffin sections of the testes, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method was employed, using an *in situ* detection kit (Promega, Madison, USA) according to the manufacturer's instructions. Samples were evaluated under a fluorescence microscope Nikon Eclipse E400, Nikon 40× Plan 40/0.65. Photographs were taken with a CCD camera VDS1300 and processed by NIS Elements AR software. In all specimens, the number of TUNEL-positive cells in 10 cross-sectioned seminiferous tubules was counted and six samples from each group were evaluated. Differences between the number of TUNEL-positive cells in the control and experimental samples were statistically analyzed.

2.10. RNA isolation

Total RNA was extracted from the left testis of experimental and control animals. For this extraction we used a Tri-Reagent kit (Sigma, Prague, Czech Republic). To each testis, 1 ml of Tri Reagent was added and the tissue was then homogenized in homogenizer Precellys 24 (Bertin Technologies, Aix-en-Provance, France). Subsequently, the samples were processed according to the manufacturer's instructions. Isolated RNA was stored at −70 °C. The RNA quality and purity were measured with a NanoDrop-1000 spectrophotometer (Thermo Scientific, Washington, USA).

2.11. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For the synthesis of cDNA, 5 μg of purified RNA was used, to which we added 1 μl DNase I (Invitrogen, Eugene, USA), 1 μl DNase I reaction buffer (Fermentas, Burlington, Canada) and H_2O to reach a volume of 10 μl. This mixture was incubated for 30 min at 37 °C in a Touchgene Gradient Thermal Cycler (Techne, Burlington, USA). After incubation, 1 μl EDTA (Fermentas) was added and incubation continued at 65 °C for 10 min. Then 30 μl of the reaction mixture (8 μl of reaction buffer for M-MuLV reverse transcriptase (Fermentas, Burlington, Canada), 5 μl 10 mM 4dNTP (Fermentas, Burlington, Canada), 0.3 μl RiboLock inhibitor (Fermentas, Burlington, Canada), 1 μl oligo (dT) + random primers (Promega, Madison, USA) and 15.7 μl H_2O) was added to the samples. The mixture was incubated for 60 min at 42 °C, for 10 min at 70 °C, and in the end was maintained at 4 °C. The obtained cDNA was stored at −20 °C.

2.12. Quantitative polymerase chain reaction (qPCR)

Gene expression analysis was performed in BioMark (Fluidigm, San Francisco, CA), which enables performing a large number of real-time PCR reactions in a single run. Before performing BioMark analysis the samples were pre-amplified. The pre-amplification reaction was done as follows: 2 μl of cDNA (10 ng RNA/μl) was mixed with 1.2 μl of 208 nM primer mix (all primers were mixed together, final concentration of each primer 25 nM), 5 μl of iQ Supermix (BioRad, Prague, Czech Republic) and 1.8 μl of H_2O. The mixture was first incubated for 10 min at 95 °C, then followed 18 cycles of 15 s at 95 °C, and finally 4 min at 59 °C. Pre-amplified cDNA was diluted 20×. The real-time PCR reactions were carried out in GE Dynamic array 48.48 in a BioMark HD System (Fluidigm, San Francisco, California). Five μl of Fluidigm sample premix consisted of 1 μl of 20× diluted pre-amplified cDNA, 0.25 μl of 20× DNA Binding Dye Sample Loading Reagent (Fluidigm), 2.5 μl of SsoFast EvaGreen Supermix (Bio-Rad, Czech Republic), 0.1 μl of 4× diluted ROX (Invitrogen, USA) and 1.15 μl of RNase/DNase-free water. Each 5 μl assay premix consisted of 2.5 μl of 10 μM primers (forward and reversed at a final concentration of 500 nM) and 2.5 μl of DA Assay Loading Reagent (Fluidigm, USA). Thermal conditions for qPCR were: 98 °C for 40 s, 40 cycles of 95 °C for 10 s, and 60 °C for 40 s. The β-actin (Actb) reference gene was selected from several reference gene candidates by Normfinder (GenEx Enterprise, MultiD Analyses, Sweden). The data were collected using BioMark 3.1.2 Data Collection software and analyzed by BioMark Real-Time PCR Analysis Software 3.1.3. (Fludigm, USA).

Table 1
Primer sequences.

Gene	Accession no.	Nucleotide sequence 5′–3′	Size of PCR product (bp)
Actb	NM_007393.3	CGGTTCCGATGCCCTGAGGCTCTT CGTCACACTTCATGATGGAATTGA	100
Ar	NM_013476.3	CCGGACCTTATGGGGACATGCG GCTGCCACAAGTGAGAGCTCCG	143
Bcl-2	NM_009741.3	CAGGGAGATGTCACCCCTGGTGG AGGCATCCCAGCCTCCGTTATCC	104
Ccnal	NM_007628.3	TTGCAGCTTGTCGGGACAGCA ACAAACTCATCCACGTCGGGCG	80
Ccndl	NM_007631.2	ACCTGGGCAGCCCCAACAAC GCCTGGCGCAGGCTTGACTC	139
Crem	NM_013498.2	GCCTCACCAGGAAG CCTGCAC TCTTCTTCCTGCGACACTCCCG	115
Ctsd	NM_009983.2	GACTCCCGGCGTCTTGCTGC AGCCGCCCACCTCCGTCATA	119
Dnmtl	NM_010066.4	AGCAAGTCGGACAGTGACACCCTTT GCCGAGTTCCCCTCTTCCGACT	149
Eps8	NM_007945.2	CCACTGCGGAGGAACGGAAGC CGTTGCGGAACCTCGGGACG	114
Fkbp5	NM_010220.3	GTTCGCAGAGCGGGACGCAA CTCCGTGGCGCAGCGTCATA	142
Fshr	NM_013523.3	GGAGCCTCTGGGCCAGTCGT GCGGTATGTTGACCTGGCCCTC	108
Grth	NM_013932.4	TACTTTGGGGAGGCGACGCC AGGTTCTTCCTGGGGTGGACGA	85
Igfbp5	NM_010518.2	CGCGGGGTTTGCCTCAACGA GGCCGGAAGACCTTGGGGGA	128
Icap1	NM_008403.4	GCCGCTGGACCATCCCACAC GCTCGATCCAGAGAAGATGCCGC	111
Kdm4a	NM_172382.2	GCAAG CTCCCACG CCACCAC CCTCGGGG GTCAGCTGCTCA	81
Mas1	NM_008552.4	CCGAGACTGCCCCAAGCCTCT TGCCCTGGGTCACTTCAGGTCAT	108
Meig1	NM_008579.4	TCTGCACTGAGTCTGGTCGTCGA TCCTCTGACCATTTCTTGGCACGA	144
p21	NM_007669.4	CCGCCGCGGTGTCAGAGTCTA CTGTGCGGAACAGGTCGGAC	120
p53	NM_001127233.1	ATGGCTTCCACCTGG GCTTCCTG CCACAACTGCACAGGGCACGT	119
Sox9	NM_011448.4	GCTGGAAGTCGGAGAGCCGAGA AGAGAACGAAACCGGGGCCAC	137
Spata2	NM_170756.2	GGGCCTGTGCTTTGGAGGCG TGGCTCTGGAACTGGAGGCTGG	115
Sycpl	NM_011516.2	GCCCATGCTCGAACAG GTTGC ACAGTCTGCTCATTGGCTCTGAA	98
Sycp3	NM_011517.2	GGACAGCGACAGCTCACCGG GGTGGCTTCCCAGATTTCCCAGA	90
Tffl	NM_009362.2	TGTCCGGGGATTCCCGTGGT CCAGTGCCCAGGTGGAGGGT	131
Tnpl	NM_009407.2	CCGAGCTCCTCACAAG GGCGT CAGGGCAGAGCTCATTGCCGC	140
Tnp2	NM_013694.4	CCTGCAAGACCCAGCCACCG GTTTCCGCCTCCTGACGGCC	94
Vegfa	NM_001025257.3	TGCTCTCTTGGGTGCACTGGAC GACGGCAGTAGCTTCGCTGGT	147
Wtl	NM_144783.2	GGCGCTTTGAGGGGTCCGAC AAAGTGGGCGGAGCACCGAC	205

Table 2
Effect of ZE on body and organ parameters in CD1 mice. Mean ± SD; $n = 18$.

Group	AGD (cm)	Body weight (g)	Testes (g)	Prostate (g)	Seminal vesicles (g)	Epididymis (g)
Control	1.57 ± 0.15	35.10 ± 2.53	0.261 ± 0.030	0.159 ± 0.030	0.310 ± 0.052	0.088 ± 0.010
High dose	1.53 ± 0.12	33.81 ± 4.30	0.239 ± 0.022	0.156 ± 0.036	0.291 ± 0.066	0.084 ± 0.006
Low dose	1.48 ± 0.07	34.63 ± 2.36	0.248 ± 0.023	0.151 ± 0.036	0.309 ± 0.041	0.087 ± 0.007

The cut-off value for Cq was set at 25 and values higher than that were replaced by the Cq value of 25. The missing data were filled with maximum of a column plus 1. Data were normalized with β-actin. The fold change in expression was calculated using the $2^{-\Delta\Delta Cq}$ method [16] for each sample and then expressed as the mean of all these fold changes. The control was set at 100% and experimental samples were compared to the control.

2.13. Selected testicular genes

Twenty-eight genes that are expressed in testicular tissue, and thus can influence the process of spermatogenesis and consequently the sperm quality, were tested. The tested genes can be divided into five groups – genes expressed in the germinal cell line (*Vegfa*, *Sycp3*, *Sycp1*, *Ccna1*, *Meig1*, *Grth*, *Prm1*, *Tnp1*, *Tnp2*), genes expressed in Sertoli cells (*Sox9*, *Wt1*, *Eps8*, *Icap1*, *Mas1*), genes playing a role in hormonal response (*Ar*, *Fkbp5*, *Tff1*, *Igfbp5*, *Ctsd*, *Fshr*), genes playing a role during apoptosis (*p21*, *Bcl*, *p53*), and genes related to epigenetic processes (*Ccnd1*, *Crem*, *Kdm4a*, *Spata2*, *Dnmt1*). Primer properties are summarized in Table 1.

2.14. Statistical analysis

Statistical analysis was performed in STATISTICA 7.0 (StatSoft, Prague, Czech Republic). The number of offspring was analyzed by the Mann–Whitney *U*-test. The body weight was compared using the analysis of variance (ANOVA), and *post hoc* analysis was performed using the Newman–Keuls test. The weights of individual organs were compared using the analysis of covariance (ANCOVA) and the body weight was used as a covariate. Data obtained from the sperm analysis and TUNEL method were analyzed using the Kruskal–Wallis ANOVA; *post hoc* analysis was performed again using Dunn's test. Statistical analysis of gene expression was performed in Genex 5.3.7 (MultiD Analyses, Sweden) using one-way ANOVA, and *post hoc* analysis was performed using the Dunnett test. A *P*-value lower than 0.05 was identified as statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Number of progeny; sex ratio; body and organ weight

Male mice were exposed to two different concentrations of ZEA during gestation, lactation, pre-pubertal and pubertal period, and up to adulthood. To evaluate the effect of ZEA on the male reproductive tract, the body and organ weight, number and sex ratio of progeny, and anogenital distance (AGD) were measured. Individuals from experimental groups were compared with control animals. This model was used in all subsequent measurements. ZEA had no effect on the number of progeny and sex ratio in both experimental groups (not shown). AGD and body weight as well as the weight of reproductive organs were not affected in these two groups (Table 2).

3.2. Sperm parameters

To evaluate the effect of ZEA on sperm quality, the sperm morphology (Fig. 1), viability and state of acrosome were evaluated. It was shown that ZEA induced a decrease in sperm quality mainly in animals exposed to the low dose of ZEA. In this group we detected significantly decreased sperm concentration (by 40%) and increased number of morphologically abnormal spermatozoa. Moreover, a significantly increased number of apoptotic spermatozoa and changes of acrosome staining were observed in this group. However, the sperm parameters of animals exposed to the high dose were also affected. We detected an increased number of apoptotic spermatozoa and changes in acrosome staining in samples obtained from this group (Table 3).

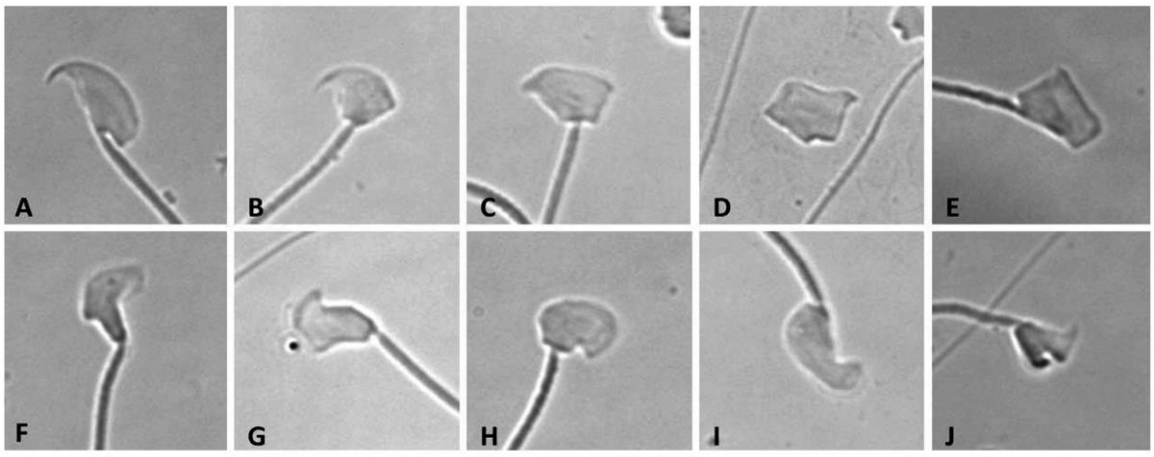

Fig. 1. Exemplary pictures from light microscopy showing sperm with normal (A) and aberrant morphology (B–J).

Table 3
Effect of ZE on sperm parameters of CD1 mice. The sperm status was analyzed by monoclonal antibodies against intra-acrosomal sperm protein (pHs-14), Hoechst 33342 (viability) and Annexin V kits. The mixture of sperm suspension was smeared onto glass slides for immunocytochemical analysis. Two hundred cells (sperm) were evaluated on each glass slide. Mean ± SD; $n = 18$.

Group	Sperm concentration ($\times 10^6$/ml)	Sperm morphology (% of normal cells)	Sperm viability (% of live cells)	Annexin V (% of positive cells)	HS-14 (% of acrosome positive cells)
Control	38.70 ± 9.35	93.60 ± 1.60	91.00 ± 1.41	61.00 ± 2.45	72.10 ± 1.96
High dose	29.90 ± 11.71	91.25 ± 3.02	89.80 ± 1.17	70.50 ± 2.26***	76.60 ± 3.58**
Low dose	23.80 ± 9.75*	91.20 ± 1.93*	89.40 ± 1.34	84.00 ± 3.16***	76.40 ± 3.98**

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

3.3. Histology of testes

Histological analysis was performed with testis paraffin sections. Morphology of seminiferous tubules and the process of spermatogenesis were evaluated visually under a microscope. In experimental groups no pathological patterns in the morphology of seminiferous tubules were observed compared to the control. Also, the process of spermatogenesis was not interrupted. In all groups, spermatogonia, spermatocytes and prolonged spermatides released into the lumen of seminiferous tubules were visible. During the examination of histological slides we did not observe any statistically significant differences in the absolute number of appropriate cell types between control and experimental groups. The group exposed to the high dose had a higher number of Sertoli cells and lower number of spermatogonia and spermatocytes compared to the control, but the differences were not significant (data not shown).

3.4. TUNEL analysis

TUNEL analysis was used to detect apoptotic cells in the testis paraffin sections. In the group exposed to the low dose we observed a slightly higher incidence of apoptotic cells, but the values did not reach the level of significance (Fig. 2). TUNEL-positive cells were observed mainly in the basal lamina of seminiferous tubules.

3.5. Real-time PCR of testicular genes

Genetic analysis was performed by quantitative Reverse Transcription Polymerase Chain Reaction (qPCR), which was performed using BioMark. The list of the tested genes (see Section 2.13) is given in Table 1.

Our experiments showed changes in the expression pattern of germinal cell-specific genes mainly in group, which was exposed to the lower concentration of ZEA. In this group we observed significantly decreased expression of the *Vegfa* gene, which is expressed specifically in spermatogonial cells. We also detected decreased expression of several genes specific for spermatocytes – *Sycp3*, *Ccna1*, and *Grth* and increased expression of gene *Sycp1*, which is also expressed in spermatocytes. Expression of the genes specific for round spermatids was not changed. In group exposed to the high dose we did not detect any significant changes in the expression of the tested germ-cell genes. The results are summarized in Fig. 3.

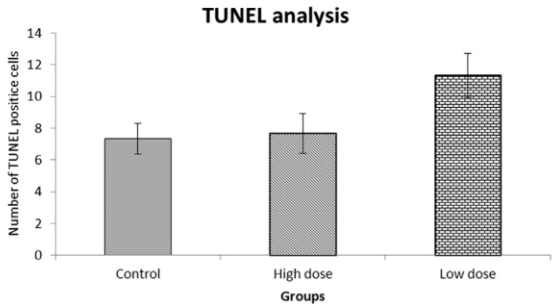

Fig. 2. Number of TUNEL-positive cells in testicular tissue sections. The high-dose group was exposed to 150 μg/l of ZE; the low-dose group was exposed to 0.15 μg/l of ZE. Mean ± SD; $n = 18$.

In the case of genes expressed specifically in Sertoli cells, we detected significantly decreased expression of *Sox9* genes in both groups and also decreased expression of *Wt1* genes in the group exposed to the high dose. In the group exposed to the low dose we detected increased expression of the *Mas1* gene. Two other tested genes (*Eps8* and *Icap1*) did not reveal any significant changes in gene expression (Fig. 4).

We detected decreased expression of the gene for androgen receptor (*Ar*) and its component *Fkbp5*. Expression of the genes playing a role in the response to oestrogen stimuli (*Tff1*, *Igfbp5* and *Ctsd*) was not changed. Expression of the gene for follicle-stimulating hormone receptor (*Fshr*) was not significantly changed (Fig. 5).

Among the tested apoptotic genes we found two genes with decreased expression, *p53* and *p21*; the decrease was observed in both experimental groups. Expression of the *Bcl2* gene was not changed (Fig. 6).

In the case of genes related to epigenetic processes, we detected significantly decreased expression of genes for Ccnd1 and Dnmt1 in the animals exposed to the low dose of ZEA and significantly decreased expression of genes for Ccnd1, Kdm4a and Spata2 in animals from group exposed to the high dose (Fig. 7).

4. Discussion

The aim of this study was to describe the influence of the mycotoxin zearalenone on the reproductive parameters and expression of selected genes in CD1 outbred mice. The tested reproductive parameters included the number of progeny in the parental generation, body and organ weight, anogenital distance, sperm quality, histopathology of the testes, apoptotic stage of testicular cells, and the expression profile of testicular genes in the offspring. Experimental animals were exposed to two different concentrations of ZEA, which was administered in drinking water. One group was exposed to the concentration of 150 μg/l and the other group to 1000 times lower concentration – 0.15 μg/l. The control group was not exposed at all. The average daily dose of ZEA for an adult man ranges from 2.4 to 29 ng/kg b.w. [2], which corresponds to our low dose (~25 ng/kg b.w.). Experimental animals were exposed starting from the first day of mothers' pregnancy up to the age of 70 days, when the animals were killed and subjected to analysis.

Our work is unique because, compared to other studies, it is focused on the effect of a low concentration of ZEA. The low concentration that was used in our experiment corresponds to the average concentration to which is exposed human population.

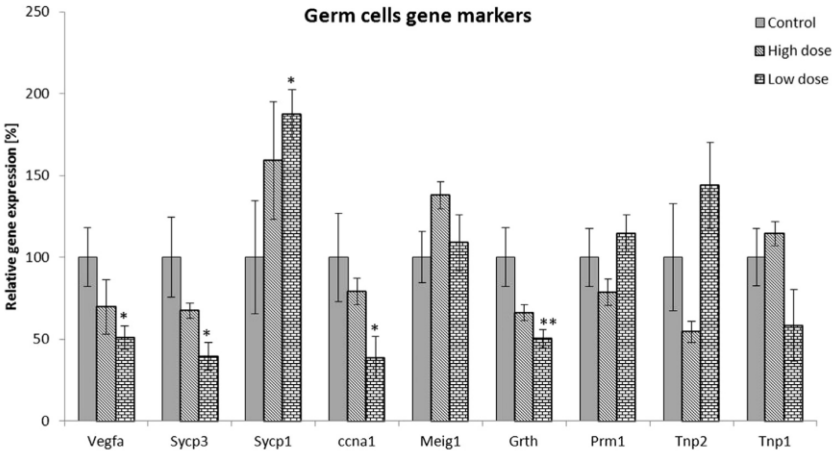

Fig. 3. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes expressed in testicular germ cells. In this study, genes for vascular endothelial growth factor (*Vegfa*), synaptonemal complex protein 3 (*Sycp3*), synaptonemal complex protein 1 (*Sycp1*), cyclin A1 (*Ccna1*), meiosis expressed gene 1 (*Meig1*), DEAD box polypeptide 25 (*Grth*), protamine 1 (*Prm1*), transition protein 2 (*Tnp2*) and transition protein 1 (*Tnp1*) were tested. The graph illustrates the relative expression of the selected genes. The control group represents 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups and the control group. Whiskers indicate the standard error. $^{*}P < 0.05$, $^{**}P < 0.01$. Mean ± SE; $n = 9$.

Animals exposed *in utero* were born with no observable pathologies; the number or sex ratio of the progeny was not affected in both concentration groups. In adult males we did not observe reduced body or reproductive organ weight, and the seminiferous tubules were morphologically normal with ongoing spermatogenesis. The first effect was observed during the evaluation of sperm parameters. We detected decreased sperm concentration, increase of morphologically abnormal spermatozoa and increased binding of apoptotic marker annexin V in the low-dose group. The decreased sperm quality and sperm count was also observed in the study by Yang et al. [10] after daily intraperitoneal injections of ZEA given for 7 days. It should be noted that the decrease in sperm concentration in the group exposed to the low concentration was almost 40%. Nevertheless, an initial concentration of about 38.7 million sperm/ml is still sufficient for reproduction. However, in case of human population where low quality of human sperm is observed, ZEA can have a significant effect on human fertility. Since the TUNEL method did not reveal any increase of apoptotic cells in the testes, and the expression of apoptotic genes was not changed in this group, it can be assumed that the reduction in the number of mature spermatozoa originated already at the level of spermatogonia. This would be in agreement with the decreased expression of the spermatogonial *Vegfa* gene (Fig. 3). The decreased number of spermatogonia after ZEA treatment was also found in the study by Filipiak et al. [13] in pubertal rats. On the other hand, two other studies have shown that ZEA is able to induce apoptosis in testicular tissue of the exposed animals [11,12]; however, in these studies much higher concentration of ZEA or different type of exposure was used.

Besides evaluation of reproductive parameters, we focused on the detection of changes in gene expression. It has been shown previously that ZEA is able to significantly influence the expression of testicular genes and thus negatively affect spermatogenesis [17]. In total we evaluated 28 genes. These genes were divided into five groups according to their function.

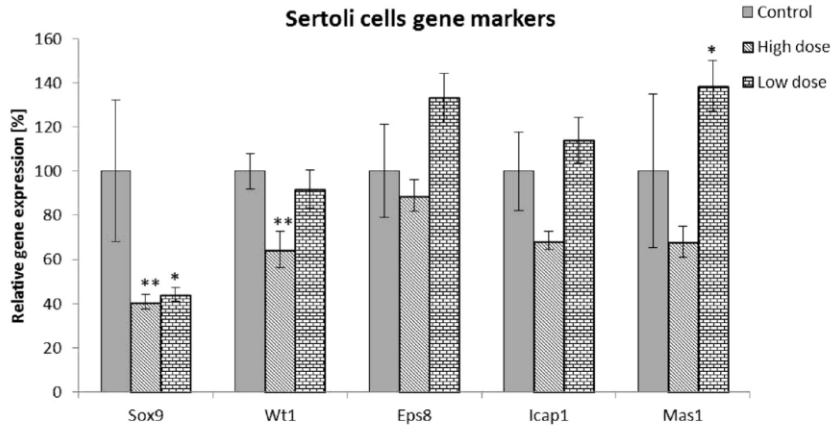

Fig. 4. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes expressed in Sertoli cells. In this study, genes for SRY-box containing gene 9 (*Sox9*), Wilms tumour 1 (*Wt1*), epidermal growth factor receptor pathway substrate 8 (*Eps8*), integrin beta 1 binding protein 1 (*Icap1*) and MAS1 oncogene (*Mas1*) were tested. The graph illustrates the relative expression of the selected genes. The control group represents 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups and the control group. Whiskers indicate the standard error. $^{*}P < 0.05$, $^{**}P < 0.01$. Mean ± SE; $n = 9$.

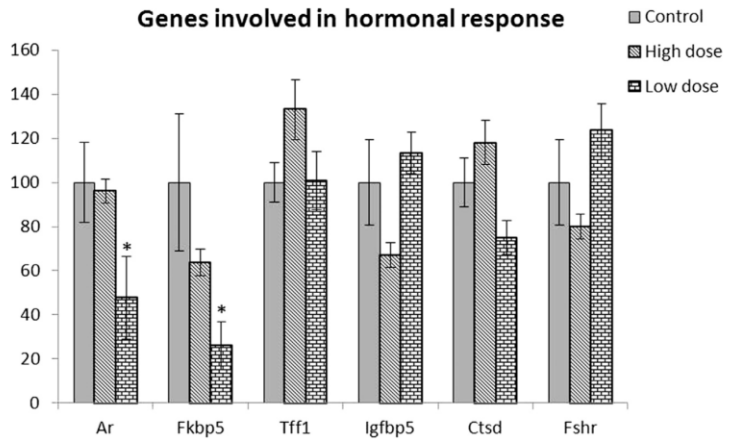

Fig. 5. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes playing a role in the response to various hormonal stimuli. In this study, genes for androgen receptor (*Ar*), FK506 binding protein 5 (*Fkbp5*), trefoil factor 1 (*Tff1*), insulin-like growth factor binding protein 5 (*Igfbp5*), cathepsin D (*Ctsd*) and follicle-stimulating hormone receptor (*Fshr*) were tested. The graph illustrates the relative expression of the selected genes. The control group represents 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups and the control group. Whiskers indicate the standard error. $^*P < 0.05$. Mean ± SE; $n = 9$.

The first group included genes regulating apoptosis – *p53*, *p21* and *Bcl2* (Fig. 6). p53 is an evolutionarily very conserved tumour suppressor protein that regulates the cell cycle, DNA repair, and apoptosis and it is also a transcription factor for *p21* and *Bax* genes. In this study we detected decreased expression of genes for p53 and p21 proteins in both experimental groups, although in the group exposed to the low dose the effect was slightly more noticeable. Since the expression of *p53* and *p21* is interrelated, it is not surprising that the change in their expression was nearly identical. The fact that these genes were downregulated indicates that ZEA had probably no effect on DNA damage. Expression of the gene encoding the antiapoptotic protein Bcl-2 was not changed, and our previous results (TUNEL assay) also did not show any changes in the number of apoptotic cells in testicular tissue (Fig. 2). A study made in fish *Kryptolebias marmoratus* has shown that short-term exposure to various endocrine disruptors (ED) such as bisphenol A or 4-nonylphenol increased *p53* expression, but after long-term exposure the expression of *p53* declined. The authors suggest that this may lead to an increased risk of potential cancer development [18].

Among further tested genes were genes expressed in testicular germ cells. The gene for protein Vegfa is expressed in spermatogonial cells and it plays a role during self-renewal and differentiation of these cells [19]. It has been shown that *in vitro* treatment of bovine testicular tissue with Vegfa results in significantly more differentiating germ cells in bovine testis and conversely, blocking Vegfa activity leads to significantly reduced numbers of germ cells [20]. Vegfa thus may support germ cell survival and sperm production. We detected decreased expression of this gene in the group exposed to the low dose of ZEA. We also tested expression of the genes specific for spermatocytes – *Sycp1*, *Sycp3*, *Ccna1*, *Meig1* and *Grth*. Sycp1 and Sycp3 are the main components of synaptonemal complex (SC) – structure playing a crucial role in synapsis and recombination during meiosis. After exposure to the low concentration of ZEA the expression of *Sycp3* decreased by almost 50% and conversely, the expression of *Sycp1* increased to 206% compared to

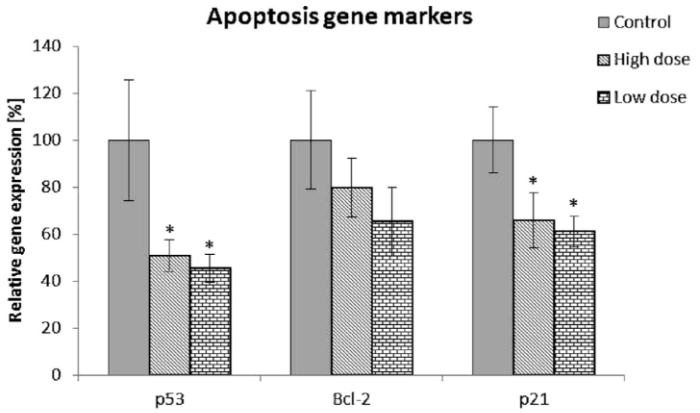

Fig. 6. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes playing a role in apoptosis. In this study, genes for tumour protein p53 (*p53*), B-cell leukaemia/lymphoma 2 (*Bcl2*) and cyclin-dependent kinase inhibitor 1A (*p21*) were tested. The graph illustrates the relative expression of the selected genes. The control group represents 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups and the control group. Whiskers indicate the standard error. $^*P < 0.05$. Mean ± SE; $n = 9$.

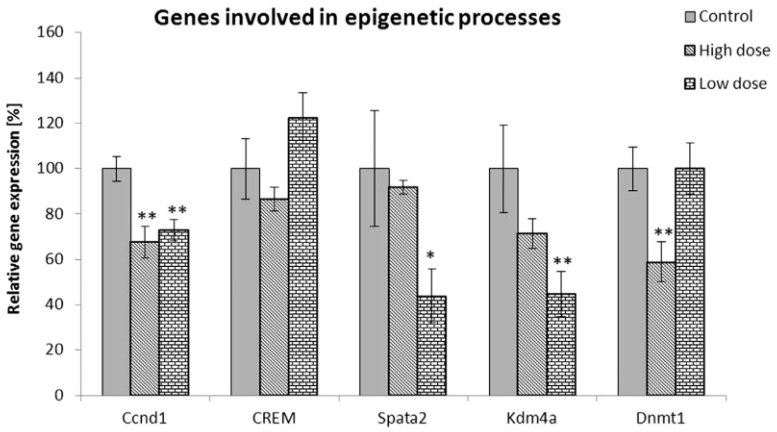

Fig. 7. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes that are related to epigenetic processes. In this study, genes for cyclin D1 (*Ccnd1*), cAMP responsive element modulator (*Crem*), lysine (K)-specific demethylase 4A (*Kdm4a*), spermatogenesis associated 2 (*Spata2*) and DNA methyltransferase (cytosine-5) 1 (*Dnmt1*) were tested. The graph illustrates the relative expression of the selected genes. The control group represents 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups and the control groups Whiskers indicate the standard error. $^{*}P<0.05$, $^{**}P<0.01$. Mean ± SE; $n=9$.

the control. However, it is unlikely that these two proteins could functionally substitute for each other because they have different functions – Sycp1 forms transverse filaments while Sycp3 forms lateral filaments of SC [21]. Recent studies have shown that Sycp1 and Sycp3 are also required for centromere pairing in the mouse, which is important for proper chromosome segregation [22]. Errors in meiotic chromosome segregation are the main cause of human aneuploidy, and therefore their altered expression, especially lack of Sycp3, could have a negative effect on the sperm quality. In the group exposed to the low dose we also detected about 50% decreased expression of cyclin A1 (*Ccna1*) compared to control. Cyclins are proteins regulating the eukaryotic cell cycle. *Ccna1* is expressed in meiotic cells and it has been shown that it is essential for spermatocytes to go through the first meiotic division. *Ccna1*$^{-/-}$ mice were sterile due to the block of spermatogenesis before the first meiotic division [23]. Mice with heterozygous mutation (*Ccna1*$^{+/-}$) were subfertile because of a low sperm count. *Ccna1*$^{+/-}$ male mice had about half the cyclin A1 protein compared to normal mice. All types of spermatogenic lineage cells were present, but the number of haploid spermatids and spermatozoa was significantly lower [24]. We can therefore say that the phenotype of *Ccna1*$^{+/-}$ mice is similar to the phenotype observed in the animals exposed to the low dose of ZEA, in which we detected about half the level of Ccna1 mRNA and nearly 40% fewer spermatozoa compared to the control, but at the same time we were able to observe ongoing spermatogenesis. We also detected decreased expression of testis-specific gonadotropin-regulated RNA helicase (*Grth*) in the testes of animals from the low-dose group. *Grth* is expressed in Leydig and germ cells (spermatocytes and round spermatids) and it is target of gonadotropin and androgen action. *Grth* knockout mice (*Grth*$^{-/-}$) suffer from azoospermia caused by spermiogenesis failure. *Grth* is an mRNA-binding protein that controls translation of mRNA whose proteins are expressed in different phases of spermiogenesis, *e.g.* transition proteins or protamines [25]. In our study we did not detect any changes of gene expression of transition proteins 1 and 2 or protamine 2 either; however, we have not tested the protein level and therefore we cannot say whether the translation was affected or not. Nevertheless, the decreased expression of *Grth* could contribute to the decreased sperm quality of animals exposed to the low dose. Taken together, it seems that ZEA may influence the spermatogonial cells – their differentiation and self-renewal – and also the spermatocytes by affecting expression of several important meiotic genes. This might be the reason for the decreased number of epididymal spermatozoa and their decreased quality in animals exposed to the low dose.

To assess the potential effect of ZEA on Sertoli cells we examined the expression profile of several Sertoli cell-specific genes (Fig. 4). One of them was nuclear transcription factor Sox9, which is preferentially expressed in Sertoli cells. In the testis of fertile one-year-old rats the Sertoli cells of some tubule segments were strongly positive for *Sox9* whereas other were negative, and further studies showed that the reaction varied systematically according to the zone or stage of spermatogenesis. In general, it is possible to say that a prominent presence of *Sox9* in the Sertoli cells is related to abundant mitosis and meiosis [26]. Decreased expression of the *Sox9* gene was detected in both groups. This effect was more obvious in the group exposed to the high dose (reduced by 38%) than in the group with low exposure (reduced by 32%) and could be caused by decreased spermatogenesis in these groups. We also analyzed expression of transcription factor *Wt1*, which is specific for foetal and adult Sertoli cells. Disruption of *Wt1* expression in adult Sertoli cells leads to interruption of apical ectoplasmic specialization (ES) (junctional complex between Sertoli cells and elongating spermatids). This can cause increased germ-cell apoptosis and reduced sperm count or motility. In mice with impaired expression of *Wt1* in Sertoli cells, dysregulation of several other genes was observed. Among these were genes for *Eps8* and *Icap1-α*, which are important signalling molecules in apical ES [27]. We detected decreased expression of the *Wt1* gene in the group exposed to the low dose (reduced by 28%); however, we did not detect any significant changes in the expression of *Eps8* and *Icap1a* genes. We therefore assume that apical ES were not substantially affected in the testes of experimental animals. Another tested gene whose expression was increased in the group exposed to the low dose was *Mas1*. *Mas1* is a G-protein-coupled receptor which is expressed in Sertoli and Leydig cells. Studies of *Mas*-knockout mice showed that lack of Mas1 affects the expression of enzymes involved in the biosynthesis of testosterone in Leydig cells. This suggests a possible role of *Mas1* in the regulation of androgen metabolism in the male reproductive system [28].

Another group of tested genes included the genes that are somehow connected with epigenetics (Fig. 7). One of them was DNA methyltransferase 1 – *Dnmt1*. *Dnmt1* has preference for hemimethylated DNA and thus is critical for maintaining

the methylation pattern during DNA replication. The protein is localized in the nuclei of all male germ cells up to pachytene spermatocytes and it is important for proper spermatogenesis [29]. We detected decreased expression of this gene in animals exposed to the high dose of ZEA. Another tested gene was the gene for lysine-specific demethylase 4A (*Kdm4a*), which specifically demethylates histone H3K9. Its expression is restricted to post-meiotic male germ cells and it is highest in round spermatids. In *Kdm4a*-deficient mice, chromatin condensation defects were observed and it has also been shown that *Kdm4a* directly binds to *Tnp1* and *Prm1* genes and thus controls their expression [30]. It has also been reported that Kdm4a interacts directly with androgen receptor (Ar) and is involved in transcription activation of target genes [31]. We detected significantly decreased expression of *Kdm4a* in the group exposed to the low dose. However, neither *Prm1* nor *Tnp1* expression was changed in this group and on the other hand, the expression of *Ar* was decreased. Here, we can only speculate whether the decreased expression of androgen receptor was linked with decreased expression of *Kdm4a* or whether it had another cause. We also tested the expression of spermatogenesis-associated protein 2 (*Spata2*). *Spata2* is expressed in Sertoli cells, where its expression is developmentally regulated. Its expression is stimulated by FSH and might also be regulated by methylation of its promoter [32]. We detected decreased expression of this gene in the group exposed to the low dose. The last tested gene was *Ccnd1* – G1/S-specific cyclin D1. Expression of the cyclin D1 gene is regulated by methylation of its promoter. Ccnd1 together with cyclin-dependent kinases 4 and 6 regulate the cell cycle transition from G1 to S phase [33]. We detected reduced expression of this gene in both treated groups. It is hypothesized that exposure to environmental pollutants may induce epigenetic changes. For example, a study of Stouder and Paolini-Giacobino [34] showed that endocrine disruptor vinclozoline is able to induce some epigenetic changes. In this study alterations in the imprinting of five paternally/maternally imprinted genes in the sperm of the offspring after administration of vinclozoline to pregnant female mice were observed. Another study showed an association between imprinting errors and azoospermia [35]. These findings indicate that epigenetics could be one of the mechanism by which the endocrine disruptors act.

We have also analyzed expression of the genes playing a role in the response to androgen (*Ar*, *Fkbp5*) and oestrogen (*Tff1*, *Igfbp5*, *Ctsd*) stimuli. In the group exposed to the low dose, we detected decreased expression of the gene for androgen receptor (*Ar*) and its component *Fkbp5*. Contrary to that, we did not detect any changes in the expression of oestrogen-responsive genes. In general, zearalenone is considered to be a substance with oestrogenic activity. This has been shown in many studies *in vitro* as well as *in vivo*. Oestrogenic activity *in vitro* has been proven by E-screen assays on MCF-7 cells [36]. In another study, authors elegantly demonstrated that ZEA acts as a full agonist to ERα-mediated transcription and a mixed agonist/antagonist for ERβ [37]. The effect of ZEA in female laboratory animals such as mice [38,39], rats [40] or pigs [41] also demonstrates its oestrogenic activity. The *in vivo* effect of ZEA on male mice is not so clear. Several studies have shown that ZEA influences the testosterone level *in vitro* [9] as well as *in vivo* [10]. Moreover, it has been demonstrated that *in utero* exposure to ZEA results in increased foetal anogenital distance, indicating an androgenic effect during foetal development [42]. In our study we did not observe any noticeable oestrogen effect of ZEA on male mice, but on the other hand we detected decreased expression of *Ar* and its component Fkbp5. Taken together with the increased expression of the *Mas1* gene and decreased expression of the *Grth* gene, we can assume that during our study ZEA also had a certain effect on the androgen hormonal system. Moreover, this idea is supported by previous studies on specific localization of ZEA to the site of testosterone synthesis in the interstitial region of the testes [43].

Animals exposed to the low ZEA concentration were affected considerably more than animals exposed to the high ZEA concentration. A similar result was observed in our laboratory for endocrine disruptor bisphenol A (BPA); the lower dose had a greater negative impact on the reproduction parameters and fertilization of mice *in vivo* [14]. BPA has been used as a model for the low dose effect and non-monotonic dose-response curve studies of endocrine disrupting chemicals. It was shown to directly bind to ER, albeit with lower affinity than natural oestrogen. It is worth noting that BPA showed both antiestrogenic and antiandrogenic effects and interfered with thyroid hormone action [44,45].

This phenomenon, when lower dosages of hormones have some effect while the high dosages have no or opposite effect, is quite common in endocrinology. There are several explanations for this phenomenon. The hormonally active endocrine disruptors interfere with endogenous hormones and act through the same mechanism, which means they bind to the receptors that subsequently mediate the response which depends on ligand concentration [46]. Also, it has been shown that at concentrations exceeding its physiological value the ligand may bind to receptors for a different hormone and induce the response [47]. Apparently different responses may therefore be observed depending on the hormone concentration.

5. Conclusion

Our study showed that a low concentration of mycotoxin zearalenone is able to negatively influence the sperm parameters and testicular gene expression of CD1 mice *in vivo*. In experimental animals we detected decreased sperm concentration (by 40%) and impairment of sperm quality (morphology and increase of apoptotic spermatozoa). Based on our experiments we can assume that the decrease in sperm concentration has its origin at the level of spermatogonia. The meiotic phase of spermatogenesis was affected by ZEA as well, and this could have caused further decrease of sperm quality. Our results have also shown that the lower dose of ZEA had a greater effect on the sperm quality and expression of important testicular genes. One possible explanation could be that zearalenone acts at the hormonal level, and it is known that a low concentration of a hormone may act stimulatingly, while high concentrations may have an opposite or no effect.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency document associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2014.01.003.

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Expression analysis of *MND1/GAJ*, *SPATA22*, *GAPDHS* and *ACR* genes in testicular biopsies from non-obstructive azoospermia (NOA) patients

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Abstract

Background: High-throughput studies provide a wide spectrum of genes for use as predictive markers during testicular sperm extraction (TESE) in combination with ICSI. In this work, we used the specimens from testicular biopsies of men with non-obstructive azoospermia who underwent TESE to investigate the expression of spermatogenesis-related genes *MND1, SPATA22, GAPDHS* and *ACR*.

Methods: Testicular biopsy specimens were subdivided into three groups: hypospermatogenesis (HS); maturation arrest (MA); and Sertoli cell-only syndrome (SCO). The levels of expression of the spermatogenesis-related genes *MND1, SPATA22, GAPDHS* and *ACR* in the testes were compared among these three groups using the reverse transcription polymerase chain reaction (RT-PCR) technique.

Results: Analysis of the expression of spermatogenic genes in human testes with abnormal spermatogenesis showed different expression patterns in patients from different groups. Fertilization rate for studied set of patients was 66% and pregnancy rate 29%. For HS group fertilization rate was 72% and pregnancy rate 32%, while for MA group fertilization and pregnancy rates were 54% and 26%, respectively. Fertilization rates in relation to the studied genes were uniformly around 70%, pregnancy rates for ACR and GAPDHS genes were surprisingly low at 6% and 8% correspondingly.

Conclusions: Analysis of the expression of genes involved in spermatogenesis can be a fast additional test for the level of spermatogenesis in testicular samples.

Keywords: Non-obstructive azoospermia, Human testes, Biopsy, Spermatogenesis, Gene expression, ICSI

Background

Testicular tissue is composed of many cell types serving as spatio-temporal environment for the male germ cell development. It is the only place in the male organism where meiosis occurs. After meiotic division, gene expression continues in haploid cells until chromatin condensation to produce proteins necessary for the final stages of spermatogenesis [1]. Germ cells also employ mechanisms for mRNA storage and delayed translation after chromatin has already been packaged. The final products of spermiogenesis are highly differentiated sperm cells, which are transcriptionally inactive. The rate of cell proliferation in testicular tissue is higher than in other tissues due to continuous sperm production. All these facts make gene expression analysis of testicular tissues extremely important.

Changes in the complex process of spermatogenesis caused by genetic background or environmental factors can lead to male infertility. Infertile men with no sperm cells in the ejaculate can father a child with the help of assisted reproduction techniques using testicular sperm. Intracytoplasmic sperm injection (ICSI) can be successful in men with non-obstructive azoospermia, but it cannot help patients with Sertoli cell-only (SCO) syndrome. Genome-wide expression studies of large groups of patients were performed to analyse the general changes in global gene expression of patients with infertility phenotypes [2-5]. This led to the identification of gene clusters

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that were differentially expressed in patients with spermatogenesis defects [4]. High-throughput studies provide a wide spectrum of genes for use as markers in the combination of testicular sperm extraction (TESE) with ICSI. Forty-seven genes exhibiting differential testicular gene expression associated with male infertility were detected in mice and 19 in humans [6]. They included genes involved in DNA repair, glutathione metabolism, proteolysis, spermatogenesis and stress response. These findings enabled the identification of markers for specific stages of spermatogenesis and the presence of somatic cells, thus improving infertility diagnostics.

In this work, we used the specimens from testicular biopsies of infertile men who underwent TESE for the ICSI procedure and investigated the expression of spermatogenesis-related genes. The *GAPDH* gene is expressed in somatic testicular cells and spermatogonia. Two genes, *MND1/GAJ* and *SPATA22*, are expressed prior to meiotic division and their corresponding proteins are involved in meiotic progression during spermatogenesis. *GAPDHS* and *ACR* genes reach the highest expression levels in haploid spermatids and are important for the sperm function.

Methods

Patients

Testicular tissue samples were collected from patients treated for infertility in ISCARE I.V.F. a.s. A total of 47 biopsy samples were obtained from azoospermic men aged 27–63 years. All patients enrolled in the study underwent testicular biopsies within their treatment and gave their written informed consent with donating the used material for the purposes of this research project. The study was approved by the institutional review board at the Institute of Biotechnology.

TESE procedure, sperm extraction and ICSI

Testicular sperm extraction (TESE) was performed as previously described [7]. Briefly, small pieces of testicular tissue were placed in a Petri dish in Flushing medium (Medicult, Copenhagen, Denmark) and cupped up using two sterile needles. The fragmented tissue was assessed for the presence of motile spermatozoa under the phase contrast microscope. The suspension of cells was cultivated for 24–48 hours before injection or freezing procedure. Prior to the sperm retrieval procedure, a small piece of testicular tissue was taken for histological examination.

TESE samples were divided into three groups: hypospermatogenesis (HS), maturation arrest (MA), and Sertoli cells only syndrome (SCO), with a histopathology score counting according to Holstein et al. [8]. The corresponding grades were 6–8 for HS, grades 3–5 for MA and grade 2 for the SCO group.

ICSI procedures were carried out according to Silber et al. [9]. The sperm cells were incubated in droplets of 5 μl of Flushing medium (Medicult) with 30% of human serum for 2 hours followed by injection into the oocyte. The fertilization rate was assessed approximately 18 h after the injection by the presence of two pronuclei and second polar body and was quantified as a percentage of fertilized mature oocytes. Clinical pregnancy was confirmed by observing the gestational sac or detecting foetal heart beats.

RNA purification

RNA purification was performed with the same piece of testicular tissue that was used for the sperm extraction and subsequently cryopreserved. Testicular biopsies with the residual medium were thawed directly in RNAlater RNA Stabilization Reagent (Qiagen, Chatsworth CA) and samples were homogenized with the Precellys 24 tissue homogenizer (Bertin Technologies, France). Total RNA was purified from the tissue samples using the RNeasy lipid tissue mini kit (Qiagen, Chatsworth CA) according to the manufacturer's instructions and stored at −70°C. The concentration and purity of the purified RNA was determined by UV spectrophotometer Helios α (Thermo Electron Corporation, Marietta, USA) and confirmed by agarose gel electrophoresis. Human total testicle RNA, 1 mg/ml (Ambion®, Life Technologies™, Carlsbad CA), was used as a positive control.

RT-qPCR

Reverse transcription and subsequent RT-qPCR was performed as previously described [10]. Briefly, prior to reverse transcription, purified RNA was treated with RNAse-free DNAse 1 (Fermentas, Burlington, Canada) for 40 min. Template cDNA was synthesized from 1 μg of total testicular RNA using SuperScript® III Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad CA) or RevertAid™ Reverse Transcriptase (Fermentas, Burlington, Canada) with combination of random hexamer and poly(dT) primers (1:1) in a Touchgene Gradient Thermal Cycler (Techne, Burlington, USA). The qPCR conditions were: initial denaturation for 15 min, followed by 40 cycles of denaturation at 94°C for 20 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Following PCR reaction, the melting curve was constructed by increasing the temperature from 72 to 95°C to ensure that the correct product is amplified in the reaction. PCR was repeated three times in doublets for each gene, and the average Ct was used for further analysis. Gene-specific primers for *PPIA*, *GAPDHS* and *ACR* were designed using the advantages of Primer3 software and BLAST alignment of Primer-BLAST service from NCBI [11]. Three best primer pairs overlapping the intron sequence were ordered and after pretesting, the best of them was used

for gene expression analysis. Due to the large amount of pseudogenes for *GAPDH* gene in human genome [12], neither of the three primer pairs ordered was suitable because of the dimer formations, and the primers used were as in Barber et al. [13]. Primers for *MND1* and *SPATA22* were as in Okada et al. [4]. The *PPIA* (peptidylprolyl isomerase A (cyclophilin A)) gene was used as a reference gene. Primer properties are summarized in Table 1.

Statistical analysis

Experimental data were analysed using STATISTICA 6.0. and GraphPad Prism 5.04. The differences between the control and experimental groups in the relative gene expression were analysed by KW ANOVA, and post hoc analysis was performed by Dunn's test. The p value that was equal to or lower than 0.05 was considered to be significant and was indicated with red asterisk in the column.

Results

A total of 47 testicular biopsies were analysed. As the specimens were primarily used for sperm retrieval, in 13 cases the level or purity of isolated RNA was not sufficient for further studies. In the remaining 34 samples, morphological examination diagnosed nine biopsies as Sertoli cell only (SCO, 26%), 12 as maturation arrest at spermatocyte stage (MA), 12 as hypospermatogenesis with few sperm cells present (HS) and one sample as obstructive azoospermia with normal spermatogenesis. A commercial total testicular mRNA was used as a positive control for the gene expression.

Table 2 summarizes individual characteristics of the *in vitro* fertilization process, numbers of fertilized oocytes, embryo transfers and the cycles as well as occurrence of clinical pregnancy. Fertilization rate for all studied samples was 63%, in particular, for HS subset - 72% and MA subset – 54%. Pregnancy rate rate was 29% for whole set of patients, from this 32% for HS group and 26% for MA group. For samples with positive expression of studied genes fertilization rate for *GAPDHS* positive subset was 66%, *ACR* - 71%, *SPATA22* – 68%, *MND1* – 70%, pregnancy rates were 8%, 6%, 18% and 36% respectively.

Testicular biopsy of OA showed a similar expression pattern to that of commercial testicular RNA (Figure 1). Three samples (10–12) from the HS group and six from the MA group (17–24) showed no or low expression of the studied genes. In the SCO group, two samples (25 and 26 in Table 2) showed decreased expression of the tested genes, whereas in the remaining seven biopsies only residual presence of GAPDHS, ACR and SPATA22 could be detected.

In patient 9 from the HS group and patients 22 and 23 from the MA group the expression of MND1 and SPATA22 was detected and no ACR or GAPDHS gene products were found.

Next, we looked whether any difference in relative expression of the studied genes could be found between the histological groups of HS, MA and SCO. Relative gene expression was significantly decreased for *SPATA22* and *GAPDHS* in the SCO group (Figure 2). The *ACR* gene was downregulated as well, but due to high inter-individual differences and the low number of studied samples in the groups the decrease was not significant.

Discussion

The main goal of all analytic procedures in patients with non-obstructive azoospermia is to quickly obtain reliable data for successful prediction of testicular sperm retrieval. Some laboratories attempted to predict spermatogenesis with non-invasive techniques with differing success [14-16]. To date, the only generally accepted reliable predictor of successful TESE is testicular histology [17]. Analysis of the germ cell-specific gene expression in testicular samples can provide an additional, supplementing approach to increase the prediction of positive TESE outcome.

Testicular transcriptome consists of gene expression patterns of both somatic and germ cells and has been intensively studied in recent years [3]. The first studies were focused on describing the global testicular gene expression and identifying testicular genes in mice [18] and human [19]. Shima et al. [1] took advantage of the first synchronous wave of spermatogenesis in pubertal mice to locate the gene products to specific testicular

Table 1 Primer sequences

Gene	Accession no.	Forward primer sequence (5' - > 3')	Reverse primer sequence (5' - > 3')	PCR product size, bp	Reference
MND1	NM_032117.3	GTTGATGATGGTATGGTTGACTGTG	CCCTCAGACAACTGAGATTCCAGA	125	5
SPATA22	NM_032598.4	TGGCGTGAACATGCACAGAA	TTCGAATAATATGGGCCAGGTGTAA	89	5
GAPDHS	NM_014364.4	AAGGGGCCCATGGCTGGCATC	GCATCGAAGATGGACGAGTGGGT	92	this MS
ACR	NM_001097.2	TTGCTAAAGATAACGCCACGTGTGA	ATTTTTGCCGACGAAGCAGTGAGC	230	this MS
GAPDH	NM_002046.4	GAAGGTGAAGGTCGGAGTCAAC	CAGAGTTAAAAGCAGCCCTGGT	71	11
PPIA	NM_021130.3	CCCACCGTGTTCTTCGACATT	GGACCCGTATGCTTTAGGATGA	275	this MS

Table 2 Fertilization outcomes in individual patients

Sample	Spermatogenesis	Oocytes	Fertilized	Ebbryos transferred	Number of cycles	Clinical pregnancy
1	HS	4	4	2	1	No
2	HS	8	5	2	2	No
3	HS	9	8	3	1	Yes
4	HS	1	1	1	1	No
5	HS	5	3	1	1	No
6	HS	12	7	2	2	No
7	HS	5	2	1	1	No
8	HS	4	4	2	1	Yes(2)
9	HS	13	11	2	1	Yes(2)
10	HS	18	12	2	2	No
11	HS	3	3	2	1	Yes/AB
12	HS	10	6	2	1	Yes
	HS total	92	66	22	15	7
13	MA	14	13	2	1	No
14	MA	8	5	4	2	No
15	MA	11	3	2	2	No
16	MA	9	5	2	1	No
17	MA	8	2	2	1	yes
18	MA	5	2	2	1	No
19	MA	8	4	4	3	yes
20	MA	5	5	2	1	Yes(2)
21	MA	5	4	NA	NA	No
22	MA	2	1	1	1	No
23	MA	9	4	1	1	Yes
24	MA	8	2	1	1	Yes/AB
	MA total	92	50	23	15	6
25	SCO	-	-	-	-	-
26	SCO	-	-	-	-	-
27	SCO	-	-	-	-	-
28	SCO	-	-	-	-	-
29	SCO	-	-	-	-	-
30	SCO	-	-	-	-	-
31	SCO	-	-	-	-	-
32	SCO	-	-	-	-	-
33	SCO	-	-	-	-	-
34	OA	4	2	2	1	No

HS – hypospermatogenesis; MA – maturation arrest; SCO – Sertoli cell only; OA – obstructive azoospermia; AB- miscarriage; N/A- data not available.

cells. In a different approach, germ cells were purified for high-throughput analysis of cell-specific gene expression studies on animal models [20,21]. The data from the above-mentioned studies provides a vast number of possible gene candidates as markers of spermatogenesis that fulfil the criteria of testis-specific gene expression, are transcribed and translated at specific time points of spermatogenesis, and their presence indicates correct gamete development. In addition, it was shown recently that besides changes in mRNA levels in azoospermic men, the miRNA expression is also altered [22].

Another approach to TESE sample analysis is to verify whether the expression of a single gene or a couple of genes can be used as a simple indicator of positive

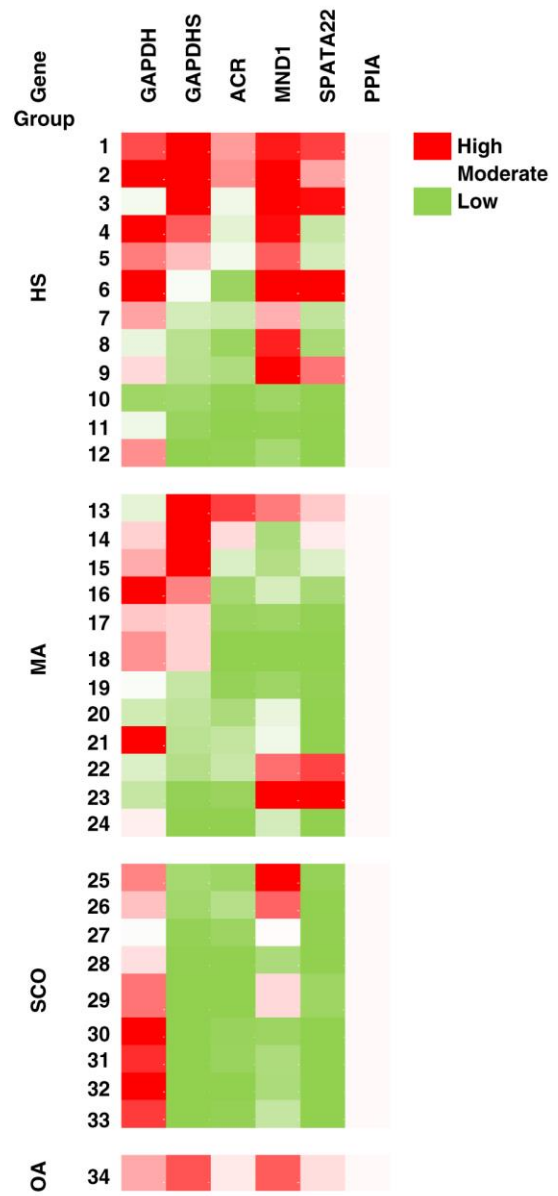

Figure 1 Heat map representing the differential gene expression of the tested genes among individual samples and groups. Red color indicates the highest gene expression (90th percentile), white color indicates moderated gene expression and green color indicates lowest gene expression (10% percentile). Other shades indicate intermediate values.

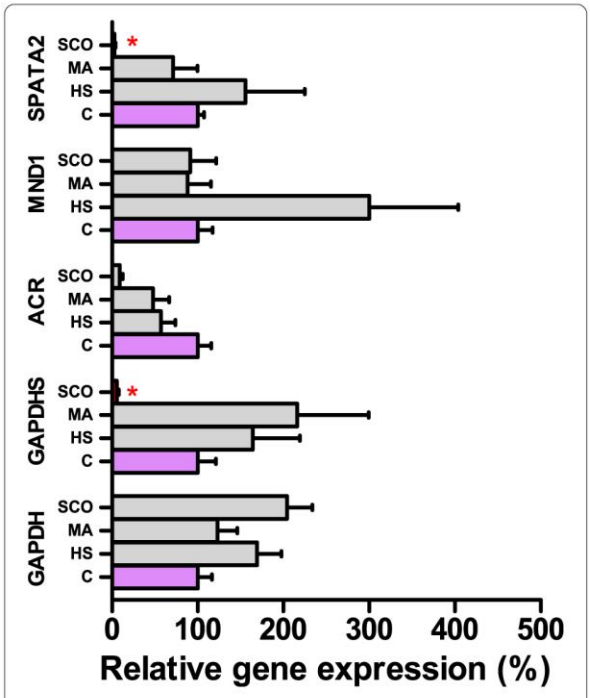

Figure 2 The relative difference in the expression of tested genes among control and experimental groups of hypospermatogenesis (HS), maturation arrest (MA) and Sertoli cell- only syndrome (SCO). Columns indicate the arithmetic mean of the relative expression of a particular gene, whiskers indicate SEM. Control group (C) is indicated by purple color and the relative expression was set as 100%. The statistically significant differences between control and experimental groups are indicated by red color of the column or red asterisk.

sperm retrieval in patients undergoing treatment in infertility clinics. Detection of DAZ (deleted in azoospermia), DAZL (DAZ-like) and protamine 2 (PRM2) mRNA in testicular samples was shown to be an informative tool for spermatogenesis evaluation [23]. Similar results were obtained with the *BOULE* mRNA occurrence [24]. Ando et al. showed that expression of *VASA*, *ODF1*, *ODF2* (outer dense fiber 1 and 2) and *SMCP* (sperm mitochondria-associated cysteine-rich protein) genes was significantly stronger in the successful TESE group [25]. Other genes expressed in post-meiotic stage may also be good candidates for the prediction of successful fertilization.

In our study, we followed expression of five genes that are expressed at certain stages of the spermatogenesis process and are important for meiosis and sperm development. The *MND1* and *SPATA22* genes were selected for spermatogenesis characterization in azoospermic patients because of the known significant gene expression differences in different non-obstructive azoospermic patients [4]. The Mnd1/Gaj protein plays an important role in homologous chromosome pairing and efficient crossover during meiosis [26]. The *SPATA22* gene product was shown to be involved in meiotic progression of germ cells in mice [27]. The *ACR* mRNA appears first in pachytene spermatocytes, reaching the maximum levels in round spermatids, and preproacrosin protein appears in spermatids [28]. The reason for non-obstructive azoospermia in this case may be interrupted or incomplete

spermiogenesis or sperm maturation. Nevertheless, the loss of acrosin protease activity does not lead to infertility in mice and spermatozoa from knock-out mice can penetrate zona pellucida of the oocyte [29]. The gene encoding sperm-specific glyceradehyde-3-phosphate dehydrogenase, *GAPDHS*, was shown to be expressed solely in haploid round and elongating spermatids [30,31] to replace the function of somatic *GAPDH* gene, whose expression ceases in germ cells. *GAPDHS* gene expression may be a good marker for spermatogenesis analysis, as its transcription and translation are temporarily separated and mRNA forms a complex with an RNA-binding protein, which results in translation and mRNA degradation delay [32]. Therefore, expression of the *GAPDHS* gene might be detectable even in poor-quality or low-quantity testicular samples. Poor detection of gene expression in nine biopsies (10–12 from HS and 17–24 from MA groups) suggests that in the tissue analysed for RNA purification, spermatogenesis was either greatly reduced or RNA was probably purified mainly from somatic cells. An interesting pattern of gene expression was observed in patients 9, 22 and 23 with normal expression of *MND1* and *SPATA22* genes and residual levels of *GAPDHS* and *ACR* genes. This might indicate that spermatogenesis in these patients continues undisturbed until meiosis, but either meiosis or spermiogenesis is somehow impaired.

Fertilization and pregnancy rates in population of studied patients were in accordance to those from previous studies. Fertilization rates for subsets of samples with positive expression of studied genes showed uniform fertilization rates around 70%, only *MND1* gene was expressed in samples from SCO group where sperm cells could not be retrieved. Surprisingly, for most promising markers of final steps of spermatogenesis, *ACR* and *GAPDHS*, pregnancy rate was below 10%. This indicates that expression analysis of present testicular genes cannot indicate successful pregnancy in studied couples. It is highly probable that in this process, oocyte and embryo quality have higher impact on the successful pregnancy. Moreover, low number of studied samples does not allow drawing any correlation between specific gene expression and fertilization outcome.

All four studied genes are expressed at different stages of spermatogenesis, and *ACR, SPATA22* and *GAPDHS* gene expression might be a good predictor of successful TESE outcome. Nevertheless, analysis of a greater number of testicular biopsies is needed to confirm that changes in gene expression of the selected genes can serve as markers to justify repeated TESE. Another thing to consider is that spermatogenesis is a dynamic process and TESE sample analysis provides information about the gene expression and spermatogenesis state at a single time point only.

A novel non-invasive approach to prediction of the state of spermatogenesis and pathophysiology of testicular tissues via the detection of germ cell-specific mRNA traces in seminal plasma was introduced recently [33,34]. Future analysis of germ cell-specific genes, including those from our study, or *GAPDH/GAPDHS* ratio in cell-free seminal plasma from azoospermic patients might become a promising non-invasive tool for TESE success prediction. The advantage of this technique is that the seminal analysis provides complex whole-testis physiology in comparison to the TESE sample representing a limited region of the analysed tissue.

To sum up, non-obstructive azoospermia is a complex pathophysiological state that leads to changes of gene expression in the testes, and understanding this process may lead to identification of the molecular markers of the spermatogenesis level.

Conclusions

Expression analysis of genes whose expression occurs exclusively in germ cells during spermatogenesis provides sensitive confirmation of the histological diagnosis of SCO syndrome, as it was decreased in all histologically identified SCO patients. In the case of maturation arrest or hypospermatogenesis, gene expression analysis could help determine the stage at which spermatogenesis arrest occurs and be a key factor in making the decision whether repeated TESE could be considered after previous ICSI failure.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AD carried out the cDNA synthesis, qPCR analysis, participated in the study design and drafted the manuscript; OT and KK collected the samples and corrected the manuscript; EZ performed RNA work and critically read the manuscript; LD participated in the design of the study and performed the statistical analysis; JP participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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Adverse effect of tetracycline and doxycycline on testicular tissue and sperm parameters in CD1 outbred mice

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ABSTRACT

Tetracycline and doxycycline are commonly used antibiotics in acne treatment during puberty in humans. The long-term effect of these antibiotics on male reproductive tract development has not been fully elucidated. For this reason we tested the effect of antibiotics on the reproductive parameters of mice males during puberty with the therapeutic dose used in humans, and with lower and higher doses. The outbred mouse strain CD1 with higher heterozygosity was exposed for 14 days at puberty. Adult males at the age of 70 days were used for the measurements. We observed a significant decrease in anogenital distance and thickness of the seminiferous epithelium in the treated animals. Pathological changes in the testes had an impact on sperm quality; a higher number of sperm positively stained with Annexin V and TUNEL and a lower number of acrosome-intact sperm was detected. In conclusion, the treatment of male mice with antibiotics in puberty led to long-lasting effects on reproductive organs and spermatozoa in adult males.

1. Background

Anthropogenic pollutants may interfere with the biological functions of the organism and, consequently, cause problems in mammalian reproduction. There are many compounds with a verified or potential adverse effect on mammalian reproduction and one group of these compounds are antimicrobial drugs. Tetracyclines represent one of the most widely used groups of antibiotics in the world. They have been used extensively for disease control and in livestock feed for several decades thanks to their great therapeutic values (Kemper, 2008; Kümmerer, 2009a). The tetracycline antibacterial group has a broad-spectrum of antimicrobial activity against a wide range of microorganisms including gram-positive and gram-negative bacteria, chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites (Chopra and Roberts, 2001). A number of biochemical dysfunctions have been associated with the long-term administration of tetracyclines (Velicer et al., 2004; Asha et al., 2007; Heaton et al., 2007; El-Hallak et al., 2008; Shelat et al., 2011). Moreover, the drug has gained wide acceptance in a lot of countries because of its easy availability.

Tetracyclines are used to treat acne vulgaris and other skin disorders such as rosacea and perioral dermatitis. Acne is the most common skin disease of adolescence, affecting over 80% of teenagers (aged 13–18 years) at some point. Oral tetracyclines are indicated for the management of severe acne, acne that is resistant to topical treatment and acne that covers large parts of the body surface. The most commonly prescribed tetracyclines are first-generation cyclines (tetracycline HCl and oxycycline) and second-generation cyclines (doxycycline, minocycline and limecycline). Second-generation cyclines have a better pharmacokinetic profile, which has been thought to be associated with increased antiacne efficacy (Healy and Simpson, 1994; Simonart et al., 2008).

However, depending on the mechanism of resistance, low-dose antibiotics may encourage overgrowth of resistant strains. For this reason, the common practice of using low-dose antibiotics for prolonged periods of time should not be recommended (Dréno et al., 2004; Simonart et al., 2008). Tetracycline is usually prescribed at a dosage of 500 mg and doxycycline at a dosage of 50–100 mg twice a day. Seven to eight years previously Tan (2003) and Feldman et al. (2004) recommended taking antibiotics for 6–8 weeks before the results are evident and treatment should be given for six months to prevent the development of microbial resistance. At present, a shorter treatment of 12 weeks is preferred (Fanelli et al., 2011; Leyden and Del Rosso, 2011; Ramanathan and Hebert, 2011).

Previous observations have demonstrated that tetracycline irreversibly reduced viability and sperm motility *in vitro* (Hargreaves et al., 1998). Damage to the reproductive organs and sperm after treatment with tetracycline has also been described *in vivo* in male

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rats (Farombi et al., 2008). In the present study we used a mouse model to investigate the effect of tetracycline and doxycycline on reproductive tract development during adolescence of males. The outbred mouse strain CD1 with higher heterozygosity was exposed to three different doses for 14 days at puberty. Half of the therapeutic dose (A), the therapeutic dose (B) and double the therapeutic dose (C) were used for the treatment. The aim was to determine whether the long-term antibiotics affect body and organ weight, reproductive parameters and sperm quality in adult male mice.

2. Methods

2.1. Animals and treatments

CD1 (ICR) outbred mice (AnLab Ltd., Prague, Czech Republic) were used for the experiments, 20 animals per group. The weaned males were randomly divided into groups of four animals, and housed in polycarbonate boxes (675 cm^2) on bedding of sterilized white pine shavings under the artificial light/dark cycle 12:12. Pelletted soya-free food without phytoestrogens (Ssniff, Soest, Germany) and water were available *ad libitum*. The animals were kept in the animal facility of the Institute of Molecular Genetics, v.v.i., Prague at a controlled temperature (23–24 °C), humidity (60 ± 5%) and ventilation (10–15 air changes/h). Antibiotics were administered to drinking water to attenuate all forms of perceived stress. Three concentrations of tetracycline (TET) and doxycycline (DOX) were selected, respectively: 7 (A), 14 (B) and 28 (C) mg/kg, bw/day of tetracycline (Tetracycline hydrochloride, Sigma, Prague, Czech Republic) and 1.4 (A), 2.8 (B) and 5.6 (C) mg/kg, bw/day of doxycycline (Doxycycline hyclate, Sigma, Prague, Czech Republic). Mice were exposed to the drugs at puberty on postnatal days 30–44 (Barnett, 2007; Pinter et al., 2007). Males without treatment were used as a control group. All animal procedures were carried out in the strict accordance with the Animal Scientific Procedure, and subjected to review by the local ethics committee.

2.2. Body and organ weight and anogenital distance (AGD)

Adult mice were killed by cervical dislocation at the age of 70 days. The weight of the body, reproductive organs (testicles, epididymides, prostate and seminal vesicles), liver, spleen, and kidneys and the anogenital distance (AGD) were evaluated.

2.3. Preparation of sperm cells

Spermatozoa from the proximal fifth region of the left and right cauda epididymis were released into 100 μl of PBS each for 5 min at 37 °C. The concentration of spermatozoa from both epididymides in 1 ml was assessed in a haemocytometer chamber according to the standard protocol under 100× magnification. Part of epididymal spermatozoa was washed twice with phosphate-buffered saline (PBS), pH 7.4, centrifuged for 15 min at 200 × *g*, and then sperm suspensions were smeared onto glass slides for immunocytochemical analysis.

2.4. Histological analysis and tissue morphometry

The right testis was fixed in Bouin solution and then processed by standard histological methods. Specimens were dehydrated, incubated in xylene and embedded in paraffin wax. Two micrometre thick slices were cut from each sample, and afterwards the slices were stained with haematoxylin–eosin. The prepared samples were evaluated using a Nikon Eclipse E600 microscope (Nikon Corporation Instruments Company, Japan). The objective evaluation consisted of measuring the seminiferous tubules. In each sample, the diameters of 50 tubules and the epithelial thickness of 50 epithelia were measured using the NIS-Elements version 3.0 software (Laboratory Imaging, Ltd., Prague, Czech Republic). The results were statistically evaluated.

2.5. TUNEL analysis

The number of apoptotic cells in tissue sections of control and experimental animals were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), using an *in situ* detection kit (Promega) according to the manufacturer's instructions. In brief, paraffin-embedded tissue sections were rehydrated in water, re-fixed in 4% formaldehyde, incubated in proteinase K solution (20 μg/ml) for 5 min, washed two times in PBS, incubated for 10 min in an equilibration buffer and finally exposed for 60 min to a labelling buffer containing both FITC-labelled dUTP and terminal deoxynucleotidyl transferase (control samples without the terminal deoxynucleotidyl transferase enzyme or treated by DNase were also prepared). Before examination, TUNEL-labelled samples were washed in saline–sodium citrate buffer and in water and mounted in Vectashield with DAPI dye to visualize the nuclei. Specimens were evaluated under a fluorescent microscope. In all specimens, the number of TUNEL-positive cells in 20 cross-sectioned seminiferous tubules was counted. Differences in the number of TUNEL-positive cells between control and experimental samples were statistically analysed.

2.6. Indirect immunofluorescence

Monoclonal antibody Hs-8 against intra-acrosomal sperm proteins (prepared in our laboratory; Peknicova et al., 2001, 2002) was used to determine the acrosomal status of epididymal spermatozoa by the immunohistochemical method. Epididymal spermatozoa loaded on glass slides were air dried and fixed for 10 min with acetone. Slides were rinsed with PBS and after that incubated overnight at 4 °C with Hs-8 antibody (diluted in PBS to an immunoglobulin concentration 20 μg/ml). After thorough washing with PBS, the smears were reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM immunoglobulins (μ-chain specific; Sigma, Prague, Czech Republic), diluted 1:128 in PBS and incubated for 60 min at 37 °C, washed with PBS and distilled water and mounted in Vectashield H-1200 DAPI (Vector Laboratories, Inc., Burlingame, CA). An Annexin V–FITC apoptosis detection kit (Sigma, Prague, Czech Republic) was used to assess the sperm damage according to laboratory instructions. Samples were examined with a Nikon Eclipse E400 fluorescent microscope equipped with a Nikon Plan Apo VC 60/1.40 oil objective (Nikon Corporation Instruments Company, Japan) and photographed with a CCD 1300-VDS camera (Vosskühler GmbH, Osnabrück, Germany) with the aid of the NIS-Elements Ar imaging software (Laboratory Imaging, Ltd., Prague, Czech Republic). Ten times 200 cells were evaluated in each group.

2.7. Flow cytometry analysis of testicular suspensions

After dissection, the left testes were placed into a glass homogenizer with 2 ml of PBS and homogenized manually to obtain single-cell testicular suspension. After homogenization, the suspension was filtered through a 70 μm nylon filter (BD Falcon, Prague, Czech Republic) to remove residues of the tough tissue. After filtration, single-cell testicular suspension was centrifuged (300 × *g*) and resuspended in 1 ml of 4% formaldehyde in PBS (pH 7.0), and the cells were fixed at RT for 60 min. After fixation, the suspension was centrifuged at 300 × *g* for 5 min, resuspended in 96% ethanol and refrigerated at −20 °C until the time of analysis. After centrifugation (3000 × *g*, 5 min) and washing in PBS (two times), the concentration of cells was determined by an automated cell

Table 1
Effect of tetracycline (TET) and doxycycline (DOX) on anogenital distance (AGD), body and selected organ weights in adult CD1 male. Mice were treated peripubertally with three different doses of antibiotics (A, B, C), and the control received no treatment. Data are means ± SEMs.

Group	AGD (cm)	Body weight (g)	Testicles (mg)	Prostate (mg)	Seminal vesicles (mg)	Epididymides (mg)	Liver (g)
Control	1.61 ± 0.1	36.4 ± 2. 5	245.7 ± 25.5	137.3 ± 34.2	277.5 ± 65.2	84.8 ± 6.9	1.9 ± 0.3
TET A	1.46 ± 0.2**	34.2 ± 1.8	215.8 ± 22.8*	136.6 ± 40.5	240.2 ± 34.7	80.3 ± 3.8	1.8 ± 0.1
TET B	1.48 ± 0.1*	34.7 ± 2.2	238.0 ± 13.7	137.4 ± 32.4	272.6 ± 46.6	80.2 ± 6.6	1.7 ± 0.2
TET C	1.57 ± 0.1	39.4 ± 2.7**	242.9 ± 24.9	147.4 ± 35.9	313.1 ± 27.4*	86.4 ± 7.2	2.3 ± 0.2***
DOX A	1.37 ± 0.1***	36.3 ± 2.1	205.8 ± 14.0*	120.8 ± 39.0	271.2 ± 51.4	75.8 ± 8.5 *	1.9 ± 0.2
DOX B	1.45 ± 0.1**	34.5 ± 2.2	249.8 ± 42.8	153.6 ± 26.2	222.5 ± 19.2*	92.0 ± 12.5	1.8 ± 0.2
DOX C	1.42 ± 0.1***	35.3 ± 2.4	225.8 ± 31.6	141.8 ± 30.5	217.0 ± 33.4*	85.8 ± 7.8	1.9 ± 0.2

* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

counter and equal numbers of cells were used for TUNEL analysis, which was performed exactly according to the manufacturer's protocol (Flow Cytometry Kit for Apoptosis, APOBRDU, Sigma, Prague, Czech Republic). After TUNEL-positive cell detection, part of the suspension was used for detection of sperm, spermatids, Sertoli and Leydig cells by specific antibodies. For sperm and round spermatid detection, the Hs-8 antibody was used (Peknicova et al., 2001, 2002). The Sertoli cells were detected with Anti-SOX9 (Abcam, Prague, Czech Republic), and Leydig cells with Anti-INSL3 antibody (Abcam, Prague, Czech Republic), both at final concentration 1 μg/ml. Cells were co-incubated with primary antibodies overnight at 4 °C. After washing (two times with PBS), suspensions were co-incubated with appropriate fluorescence-labelled secondary antibodies: Alexa Fluor 555 goat anti-mouse IgM antibody (Invitrogen, Prague, Czech Republic), for Hs-8 antibody, and Alexa Fluor 555 goat anti-rabbit IgG antibody (Invitrogen, Prague, Czech Republic), for SOX9 and INSL3 antibodies, for 60 min at 37 °C. After washing (two times with PBS), 300 μl of suspensions were co-incubated with propidium iodide (final concentration 3 μM). The suspensions were placed onto a 96-well plate, each in three wells in 100 μl volume, and analysed with a BD LSR II instrument (BD, Becton Drive Franklin Lakes, NJ, USA). In the first gating step, the population of the cells was isolated from the cell debris. After the first procedure, the three populations of cells according to their ploidy (haplo, diplo and tetraplo gates) were separated by gating in the PI channel. Gates of individual cell populations were analysed to determine the relative number of Sertoli and Leydig cells (diploid gate), sperm and spermatids (haploid gate) and TUNEL-positive cells (haploid, diploid, tetraploid gates and all gates together). In all wells, 10,000 cells (incidents) were counted.

2.8. Statistical analysis

The statistical differences among the compared groups were analysed using STATISTICA 7.0. (Statsoft, Czech Republic). When using parametrical tests, the normal distribution of the data was tested. Differences between AGD, body weight, sperm concentration, seminiferous epithelium thickness and tubule diameter were analysed by one-way ANOVA and the Newman–Keuls tests. Differences between organ weights were tested by ANCOVA with body weight as covariate. Differences between sperm parameters and number of apoptotic cells in tissue sections were assessed by KW ANOVA and multiple comparisons of mean ranks. The P value <0.05 (<0.01, <0.001, respectively) was considered to be significant.

3. Results

3.1. Body and organ weight

The body and organs of the sacrificed mice were examined. First, the anogenital distance was measured. According to our results, this distance was reduced in all experimental groups, and the reduction was significant in the groups treated with two lower doses of tetracycline and all doses of doxycycline. The antibiotics also generally decreased the body weight of experimental animals, but the reduction was statistically significant only in animals influenced by the highest dose of tetracycline. Considering organ weights, the lowest doses of both antibiotics significantly reduced the testis weight. Higher doses did not display such effect. The weight of the prostate did not express a significant change; the effect on seminal vesicles and epididymidal weight differed among the two antibiotics and their doses (Table 1). Tetracycline significantly increased the weight of the liver in its higher dose (Table 1) and did not affect the spleen and kidneys.

3.2. Spermatozoa status

Considering the concentration of sperm in the epididymides, both antibiotics significantly decreased the concentration of sperm in the highest dose, but the second higher dose of doxycycline had the most significant effect. The sperm morphology was not significantly influenced by any concentration of both antibiotics. Tetracycline had no effect on sperm viability in comparison to doxycycline, which significantly decreased the number of viable sperm in the two higher concentrations. The number of cells positively stained by apoptotic marker Annexin V was significantly influenced by both antibiotics. In the case of tetracycline, the two higher doses significantly increased the number of positive cells; doxycycline significantly increased this number in all doses, and the highest dose also had the highest effect on this parameter. Both antibiotics had no effect on the number of acrosome-intact epididymal sperm detected by the Hs-8 antibody, except for the highest dose of doxycycline (Table 2).

3.3. Histology of testes

During histological analysis, a concentration-dependent reduction of the seminiferous epithelium thickness was observed in animals influenced by the two higher doses of both antibiotics (Figs. 1 and 2). Simultaneously, no reduction of the diameter of seminiferous tubules was observed. The number of TUNEL-positive apoptotic cells was significantly increased in all experimental groups with the exception of animals influenced by the lowest dose of tetracycline. The number of apoptotic cells positively correlated with the dose of both antibiotics; the most significant effect was observed with the highest dose of doxycycline, where the number of apoptotic cells was almost two times higher than the control (Fig. 2).

3.4. Flow cytometry of testicular suspensions

During flow cytometry analysis, the concentration of sperm, spermatids, Sertoli and Leydig cells was detected by specific

Table 2
Effect of tetracycline (TET) and doxycycline (DOX) on epidydimal sperm parameters. Adult CD1 mice were treated peripubertally with three different doses of antibiotics (A, B, C), and the control received no treatment. Data are means ± SEMs.

Group	Sperm concentration (10^6/ml)	Sperm morphology (% normal)	Sperm viability (% live cells)	Annexin V (% positivity)	Hs-8 (% positivity of acrosome)
Control	74.0 ± 16.4	97.0 ± 0.9	87.0 ± 2.0	38.7 ± 1.8	88.3 ± 2.5
TET A	59.6 ± 15.0	97.5 ± 0.5	85.3 ± 1.3	40.8 ± 1.8	87.9 ± 3.2
TET B	58.2 ± 3.9	97.1 ± 0.6	82.0 ± 1.4	43.8 ± 1.6**	90.5 ± 3.1
TET C	42.8 ± 19.0**	96.5 ± 0.8	82.5 ± 2.7	42.1 ± 2.8*	91.1 ± 2.5
DOX A	61.5 ± 23.1	97.5 ± 0.8	81.8 ± 2.1	45.0 ± 3.6***	91.4 ± 2.3
DOX B	28.0 ± 4.0**	96.4 ± 1.7	80.3 ± 1.0*	42.6 ± 2.6**	91.8 ± 2.4
DOX C	38.6 ± 19.3**	97.1 ± 0.6	73.3 ± 5.7***	48.6 ± 2.4***	72.8 ± 4.6***

* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

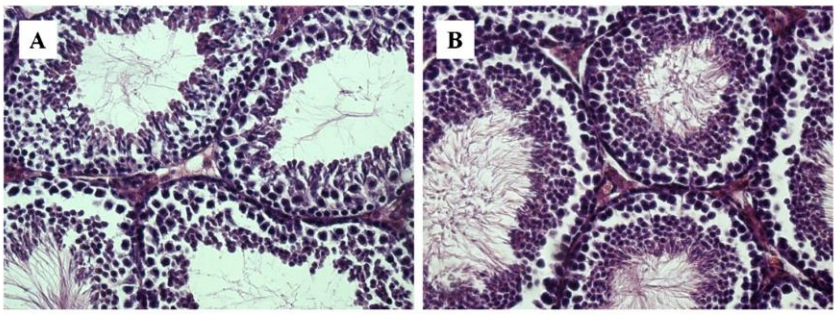

Fig. 1. Representative image of histological sections of the seminiferous tubules in adult male mice treated peripubertally with doxycycline (A), and the control received no treatment (B). In the sample influenced by doxycycline, the decrease of spermatogenesis and the expansion of the tubules with coincident impairment of germinal epithelium are noticeable. The specimen was stained with haematoxylin–eosin, 400× magnification.

antibodies. Also, the relative abundance of testicular apoptotic cells with different ploidy was assessed by propidium iodide DNA labelling and TUNEL. The number of testicular sperm and spermatids was lower. The most significant difference was detected between the control group and experimental group DOX C. Also the differences between the control and groups TET A, C and DOX B were significant. Furthermore, in the doxycycline group the effect seemed to be concentration-dependent. The concentration of Sertoli cells detected by anti-SOX9 antibody was significantly reduced only in the experimental group influenced by the highest concentration of doxycycline. The relative abundance of Leydig cells detected by anti-INSL3 antibody was not changed in any experimental group. The concentration of apoptotic cells in the entire testicular suspension followed the data from the TUNEL assay of histological sections, but the flow cytometry analysis had higher statistical sensitivity compared to the histological analysis. The effect of both studied antibiotics on the number of TUNEL-positive cells in the testicular tissue was positive and concentration-dependent. Considering the effect on cells with different ploidy, both tetracycline and doxycycline affected the haploid and diploid cell populations in the testes similarly and their effect was also concentration-dependent. On the other hand, only doxycycline had a significant effect on the incidence of TUNEL-positive cells in the tetraploid cell population (Table 3).

4. Discussion

We tested the effect of long-term tetracycline and doxycycline antibiotic administration on the reproductive tract development with a special focus on the body and organ weight, histopathology of the testes and sperm parameters in CD1 adult males. Experimental mice were treated during the puberty. This is the time of dramatic endocrine changes that are required for sexual maturation. For our study, we selected the CD1 outbred mouse strain with a higher genetic heterozygosity.

Table 3
Flow cytometry analysis of testicular suspensions in adult males treated peripubertally with tetracycline (TET) and doxycycline (DOX) in three different doses (A, B, C), and the control received no treatment. Data are means ± SEMs.

Group	HS-8 positive cells (sperm and spermatids)	SOX9 positive cells (Sertoli cells)	INSL3 positive cells (Leydig cells)	TUNEL-positive cells			
				Total	Haplo gate	Diplo gate	Tetraplo gate
Control	1842 ± 35	198 ± 6.6	155 ± 5.2	349 ± 7.1	145 ± 4.0	137 ± 4.1	67 ± 4.5
TET A	1784 ± 33*	180 ± 7.0	161 ± 4.9	359 ± 6.5	149 ± 4.7	141 ± 5.1	70 ± 5.3
TET B	1801 ± 31	183 ± 7.4	157 ± 5.2	372 ± 7.9**	151 ± 5.6*	146 ± 5.0*	75 ± 6.2
TET C	1750 ± 29**	185 ± 5.8	151 ± 4.9	378 ± 8.2***	156 ± 4.9***	151 ± 6.9***	74 ± 5.5
DOX A	1819 ± 38	190 ± 7.0	161 ± 5.8	360 ± 5.9*	145 ± 3.9	136 ± 5.0	79 ± 5.1*
DOX B	1755 ± 39*	193 ± 4.9	155 ± 5.5	422 ± 8.1***	178 ± 6.9***	165 ± 5.5***	79 ± 4.9*
DOX C	1712 ± 42***	171 ± 7.2**	163 ± 5.0	442 ± 11.7***	187 ± 7.2***	178 ± 6.7***	77 ± 4.8*

* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

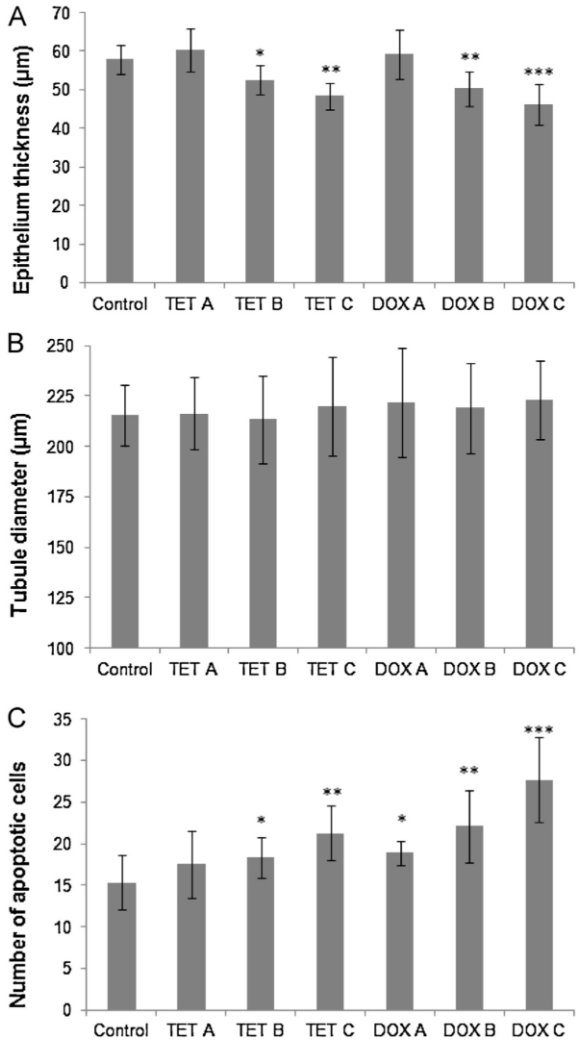

Fig. 2. Histological, morphometrical and TUNEL analysis of the testicular tissue in adult male mice treated peripubertally with three different doses of tetracycline (TET: 7 (A), 14 (B) and 28 (C) mg/kg/day), and doxycycline (DOX: 1.4 (A), 2.8 (B) and 5.6 (C) mg/kg/day), and the control received no treatment. Data are from the measurement of epithelium thickness (A) tubule diameters (B) and the number of apoptotic cells detected by TUNEL assay (C) in control and experimental samples. Data are means ± SEMs. The P value < 0.05* (<0.01**, <0.001***, respectively) was considered to be significant.

There is a growing trend towards investigation of the effects of tetracycline family antibiotics on the male reproductive organs and spermatozoa. Already in 1967 it was shown that tetracycline binds to sperm cells (Ericsson and Baker, 1967). Hargreaves et al. (1998) demonstrated that tetracycline at concentrations as low as 2.5 mg/ml led to a significant dose-dependent inhibition in the percentage of rapid-moving spermatozoa, mean path velocity, straight-line velocity and curvilinear velocity, but at 50 mg/ml tetracycline all spermatozoa were static after the capacitation process. Authors suggest that tetracycline can chelate Ca^{2+} ions, and thus changes in Ca^{2+} distribution can be followed during capacitation. Chelating Ca^{2+} ions could well be responsible for the deleterious effect of tetracycline antibiotics on mature spermatozoa. The effect of this drug was mostly irreversible. In their *in vivo* study of inbred rats, Farombi et al. (2008) have shown the second possible mechanism of tetracycline effect on the male reproductive tract – the ability of tetracycline to induce oxidative stress and its testicular toxicity. The increased levels of oxidative stress expressed by lowered enzyme activities could damage the sensitive and complex process of spermatogenesis. Authors demonstrated that a therapeutic dose of tetracycline resulted in reduced epididymal sperm motility, percentage of live spermatozoa and sperm count, increased abnormal sperm morphology, and induction of histopathological changes in the testes.

In our study we tested the effect of three doses of tetracycline and doxycycline on sperm parameters in outbred mice. The doses of both antibiotics included a therapeutic dose for acne, one lower and one higher dose. The general aim of the study was to emulate the situation in human population, and thus animals were exposed to the drugs during puberty and analysis of the effect was performed in adult fertile male animals.

In the first series of experiments we observed reduction of AGD in all experimental animals except for animals influenced by the highest concentration of tetracycline. The AGD is a highly significant marker (Hotchkiss and Vandenbergh, 2005; Gyekis et al., 2010; Eisenberg et al., 2011) of the physiological development of male reproductive tract and the reduction of this specific distance is mainly caused by a negative effect of the tested compounds on male reproductive tract development. Although the development of anogenital distance occurs in the uterus, a significant reduction of AGD was observed after pubertal exposure to di-(2 ethylhexyl) phthalate and flutamide (Vo et al., 2009). The fact that the highest dose of tetracycline (group TET C) did not result in any reduction may be caused by the activation of detoxication ability of the liver caused by a toxic dose of the antibiotic (Table 1). In this group (TET C) the weight of the liver was significantly higher compared to the control, leading to a higher body weight (Table 1). The weight of the testes was significantly lower in animals influenced by the lower doses of both antibiotics. The fact that higher doses of the drugs did not have such an effect is most likely caused by a higher amount of interstitial tissue and swelling of the organ. The weights of other reproductive organs expressed no or minute changes.

Another major goal of our investigation was to assess the sperm quality with a focus on both quantitative and qualitative parameters of the epididymal sperm cells (Table 2). The concentration of epididymal sperm was significantly lower in animals influenced by the highest concentration of both antibiotics, but the therapeutic dose of doxycycline had the greatest effect. The data from this assay are in correlation with the subjective evaluation of sperm production in testicular tissue and also with the data from seminiferous epithelium morphometry and TUNEL (Figs. 1 and 2). Evidence from all accounted methods thus supports the fact that both antibiotics had a negative effect on testicular tissue sperm production. Also, the sperm morphology parameter was almost the same in the control and experimental groups; sperm viability was lower in experimental animals and this result was highly significant in the group influenced by the highest dose of doxycycline. This effect was again in correlation with the sperm concentration and may suggest that the apoptotic process in testicular tissue is not able to remove all sperm with cellular pathology. An even better confirmation was brought by the Annexin V assay, where almost all experimental groups except for the group with the lowest dose of tetracycline (TET A) displayed a higher number of sperm stained by this apoptotic marker. This result further depicts the high activity of the apoptotic process in the testicular tissue and its inability to completely remove defective sperm, which are later present in the epididymis (Table 2).

In the next series of experiments, histological analysis of the testicular tissue showed significant differences in the epithelium

thickness, tubule diameter, apoptotic state and number of specific pathologies. The reduction of the germinal epithelium thickness is a significant marker of the developmental or pathological disintegration of the germinal epithelium (Figs. 1 and 2). We observed reduction of this parameter in animals influenced by the therapeutic and highest dose of both antibiotics. The reduction of the epithelium thickness was not in correlation with tubule diameters, and thus the reduction was caused by the process in the germinal epithelium itself. This fact is further supported by a higher number of apoptotic cells present in the tissue sections of the experimental animals. The number of apoptotic cells in the seminiferous tubules correlated with the epithelium thickness, and thus we can conclude that the reduction of seminiferous epithelium thickness is caused by pathological processes in the germinal epithelium leading to the cell death by apoptosis and necrosis. The fact that a higher number of cells undergo apoptosis suggests that this state is caused by irreversible developmental damage to the testicular tissue during exposure to the drugs in puberty.

The last series of experiments was focused on flow cytometry analysis of testicular suspensions. During these experiments, concentration and relative abundance of sperm, Sertoli cells, Leydig cells and TUNEL-positive cells were assessed (Table 3). The concentration of sperm and spermatids detected by the Hs-8 antibody was lower in all experimental groups compared to the control. The same effect was observed during analysis of the epididymal sperm, but the analysis by flow cytometry was more sensitive. The effect of doxycycline was concentration-dependent. These results are in accord with subjective histological analysis, where the decrease in sperm production especially in animals influenced by the highest dose of doxycycline was obvious, and these observations together indicate the negative effect of doxycycline on sperm production. The concentration of Sertoli cells was changed only in the group DOX C, indicating that neither antibiotic probably displayed a major effect on this cell type. The effect of the highest concentration of doxycycline could be caused by higher bioaccumulation of this antibiotic in the Sertoli cells, due to its higher lipophilicity than tetracycline (Leyden and Del Rosso, 2011) and the presence of lipid droplets in the Sertoli cell cytoplasm. Neither antibiotic had any significant effect on the concentration of Leydig cells. The fact that AGD was significantly changed despite the numbers of Leydig cell remaining unchanged could be interpreted as the restoration of Leydig cell numbers after the end of tetracycline and doxycycline administration.

Both antibiotics had a significant effect on the number of TUNEL-positive cells in the testicular tissue detected by flow cytometry. This observation largely supports the data from TUNEL analysis of histological sections. Both antibiotics had a concentration-dependent effect on the number of TUNEL-positive cells in the testicular suspensions. This fact indicates that the adverse effect of both tetracycline and doxycycline especially on the concentration of spermatids and sperm could be largely based on the pro-apoptotic effect of these antibiotics. Considering the effect on different cell types, both antibiotics seem to have a similar effect on the haploid and diploid testicular cell populations. Contrary to that, only doxycycline had a significant effect on the number of TUNEL-positive cells in the tetraploid cell population.

In conclusion, this study showed that in addition to the well-known immediate effect of tetracycline antibiotics on the reproductive system of mammals, some deleterious effects can last long after the antibiotic exposure. Previously, it was shown that immediately after tetracycline exposure, there was decrease in testicular and epididymal weight, sperm counts, and increase in oxidative stress in rat males (Farombi et al., 2008). Our results of sperm quality analysis, histological examination and TUNEL analysis suggest that spermatogenesis in mice is not fully restored even in adulthood after pubertal antibiotic exposure. The exposed animals had thinner seminiferous epithelium, increased numbers of apoptotic somatic and germ cells in the testes, and decreased numbers of epididymal sperm cells. Thus, as recent studies report increased concentrations of tetracycline in water and food (Kemper, 2008; Kümmerer, 2009a, 2009b), this may contribute to the decrease of male fertility in mammalian population.

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Effect of tetrabrombisphenol A on induction of apoptosis in the testes and changes in expression of selected testicular genes in CD1 mice

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ABSTRACT

Tetrabromobisphenol A (TBBPA) is a substance widely used in industry as a flame retardant. TBBPA was found in the environment and was detected even in the human body. The effect of this chemical was observed in different cell lines in vitro and it is supposed that TBBPA may affect various hormonal systems in vivo. In this study we examined the effect of TBBPA on the reproductive parameters of two generations of outbred mice in vivo. Experimental and control animals of F1 generation were bred in various conditions to enable evaluation of the possible trans-generational effect. An increased incidence of apoptosis in the testes and changes in the morphometry of seminiferous tubules was detected in the experimental animals. In addition, changes in the expression pattern of selected genes encoding proteins that play an important role during spermatogenesis were observed. In contrast, sperm quality and reproduction were not affected by TBBPA.

1. Introduction

Every day, numerous environmental pollutants get into our environment. These compounds, which are not naturally present in nature, can harmfully influence wildlife and the human population. Many of them can act as so-called endocrine disruptors which can disturb the physiologic function of endogenous hormones [1]. Frequently, they act as weak oestrogenic, antioestrogenic or antiandrogenic compounds, and it has been shown that they can affect both male and female reproductive development and function [2–5].

An inherent group of these compounds is represented by flame retardants. The most important and widely used group of flame retardants are brominated flame retardants (BFRs) [6]. Their production is steadily increasing [7]. Due to their structure and bromide substituents, many of BFRs are persistent, lipophilic and have been shown to bioaccumulate [8,9]. They were found in the environment far away from their place of use [10–12]. Due to these facts, BFRs have become a cause for concern as potential endocrine disruptors.

Among many BFRs, there are four groups with the highest consumption; these are polybrominated biphenyl ethers, hexabromocyklododecane, polybrominated biphenyls and tetrabromobisphenol A (TBBPA) [13]. Tetrabromobisphenol A with its global consumption of 210,000 tonnes per year is a widely used BFR [7], and was therefore selected for this study. The primary use of TBBPA is as a reactive flame retardant in epoxy and polycarbonate resins that are used in the production of circuit boards. About 10% of TBBPA has additive applications in several types of polymers [14]. When used as a reactive component, TBBPA is incorporated into the structure of the polymer and is hard to release. However, when used as an additive component, TBBPA is not part of the polymer structure and can be released more easily [6]. Nevertheless, both forms have been shown to distribute TBBPA and its derivates into their surroundings [15,16].

TBBPA has been observed in several localizations in the environment. The most common sources of emission are effluents from factories producing BFRs [16]. TBBPA has also been found in river and marine sediments in Japan [17] and sewage sludge in Sweden and Canada [16,18]. It has been detected even in air and dust samples, for example in the indoor air of electronic recycling plants and in other work environments [19]. TBBPA was detected in the air and dust of two houses in Japan and it was proven that it is preferentially adsorptive to dust [20]. TBBPA was also found in the interior dust of a television cabinet [21]. It is likely that dust plays

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an important role in human exposure to TBBPA, which confirms the findings of Jakobsson et al. [22], who investigated a group of computer technicians and detected TBBPA in 8 out of 10 blood samples. TBBPA can also be transported through the placenta to the foetus and has been found in the umbilical blood of humans [23].

The effects of TBBPA on the organism could be miscellaneous. It was shown that TBBPA can bind strongly to the thyroid hormone transport protein – transthyretrin (TTR) in vitro with an even higher affinity than thyroxin [24]. The function of TTR is to transfer thyroxin and vitamin A. TBBPA was also observed to inhibit the binding of triiodthyronine to the thyroid hormone receptor and stimulate proliferation of GH3 cells (TH-dependent pituitary cell line) [25,26]. Kitamura et al. [27] reported that TBBPA disrupts amphibian metamorphosis, which is stimulated by thyroid hormone. Beside the thyroid hormone system, even the oestrogen hormone system can be affected by TBBPA. It was shown that TBBPA or its metabolites can bind to an oestrogen receptor in vitro [28] and induce proliferation of oestrogen-dependent MCF-7 cells [29,30] or Mit/E2 cells [25].

Not much is known about the effect of TBBPA on the mammalian reproduction system in vivo. The study of Van der Ven et al. [31] performed on Wistar rats confirmed that TBBPA can interact with the thyroid hormonal system in vivo. During this experiment, the levels of circulating thyroxin, one of thyroid hormone forms, were found to be decreased. TBBPA could thus negatively influence development of the offspring because the normal level of the thyroid hormone is essential for foetal and neonatal development. Rats exposed to TBBPA also displayed increased gonad weight and plasma levels of testosterone in F1 males. These observations suggest that TBBPA could affect the androgen hormonal system as well [31]. Taken together, the findings indicate that TBBPA can negatively affect the entire mammalian reproduction system. Also its structural similarity to endocrine disruptor bisphenol A, which has been shown to negatively influence the mouse reproductive system [3], suggests its similar effects. In view of these concerns, we decided to perform a multigenerational reproductive-toxicological study to assess the effect of TBBPA on the reproductive parameters of mice during long-term exposure. This study is focused on the evaluation of male gonadal pathology, sperm quality and expression of selected genes aimed to assess how TBBPA may affect the male reproductive parameters.

2. Materials and methods

2.1. Animals and treatment

For our experiment we used the CD1 outbred mice strain (An Lab Ltd., Prague, Czech Republic) with high heterozygosity and an average litter size (12–13 pups/litter). Mice (experimental and control groups) were kept under standard experimental conditions (constant temperature (23–24 °C), humidity (60 ± 5%) and 12-hour-light regime) in the breeding facility of the Institute of Molecular Genetics, v. v. i., Prague. Animals were fed by soy-free feed (Ssniff, Soest, Germany). This type of food was used to avoid any additional influence on the animals by phytooestrogen (genistein), which is present in soy. The diet and water were administered ad libitum and all stress factors were reduced to a minimum. Experimental groups were treated with a low dose of TBBPA (Fig. 1), (Sigma, Prague, Czech Republic), which was dissolved in drinking water. The concentration of TBBPA in water was 200 μg/l. The daily dose of water consumed by the mice was calculated (5 ml/day) to expose them to 1 μg of TBBPA/mouse/day, which is equivalent to 35 μg/kg. In our experiment, the following groups were evaluated: parental animals were bred to form F1 generation. In the parental generation, control animals were not exposed to TBBPA, and in the experimental group only females were exposed during gestation. In F1 generation there were two sub-groups – **C** (control group) and **T** (group exposed to TBBPA). In group T, the pups were exposed to TBBPA during gestation, lactation, pre-pubertal and pubertal period, and up to adulthood. In group C, the pups were not exposed to TBBPA at all. F1 generation animals were bred up to the age of 70 days as follows – mother and father from group **C** formed the group **CC**; both parents from group T formed group **TT** (interbreeding), mother from group C and father from group T formed by outcrossing group **CT** and mother from group T and father from group C formed by reverse outcrossing group **TC**. Pups of F2 generation were exposed to TBBPA only in groups TC and TT (group TC was not exposed to TBBA) (Fig. 1). Animals were killed at the age of 70 days and subjected to analysis. In each group 20 animals were analyzed.

2.2. Anogenital distance, body and organ weight

Animals in the control and experimental groups were killed at the same age of 70 days. Anogenital distance (AGD) and body weight were measured and the reproductive organs were dissected and weighed. Left and right testes, epididymis, seminal vesicles and prostate were separated, cleaned and weighed individually.

2.3. Preparation of cells

Spermatozoa were obtained from proximal fifth of the left and right cauda epididymis and released to 100 μl of warmed (37 °C) PBS (phosphate-buffered saline) each for 10–15 min at 37 °C. The concentration of spermatozoa was evaluated in a haemocytometer chamber under 100× magnification. Part of the epididymal spermatozoa were taken for assessing viability, morphology and apoptotic stage. The rest of the suspension was washed twice in PBS, centrifuged for 15 min at 200 × g and smeared onto glass slides for immunocytochemical analysis. Two hundred cells (sperm) were evaluated on every glass slide; number of analyzed animals in each group was 20.

2.4. Sperm morphology and viability

To assess the morphological state of spermatozoa, 10 μl of sperm cell suspension was placed onto a glass slide, fixed at 37 °C and labelled according to the protocol using Spermac Stain System (Ferti Pro, Beernem, Belgium). Another method used for evaluation of the sperm morphological status was indirect immunofluorescence with specific monoclonal antibodies (Hs-8 and Hs-14) against intra-acrosomal proteins (see lower). Antibodies against intra-acrosomal proteins in combination with nuclei staining allow assessment of the morphology of sperm head and acrosome under the fluorescence microscope [32,33]. To determine the viability of epididymal spermatozoa a Live/Dead sperm viability kit was used (Invitrogen, Eugene, USA) according to the laboratory manual. After incubation, 10 μl of suspension was placed onto a glass slide and evaluated under a Nikon Eclipse E400 fluorescence microscope using a 40× Nikon Plan Fluor 40/0.75.

2.5. Indirect immunofluorescence

Monoclonal antibodies against intra-acrosomal proteins (Hs-8 and Hs-14) prepared in our laboratory are routinely used to test the acrosome state [32,33]. Epididymal spermatozoa loaded on glass slides were fixed for 10 min with acetone. After rinsing with PBS, the slides were incubated overnight at 4 °C with monoclonal antibodies (diluted to an immunoglobulin concentration of 20 μg/ml). After thorough washing with PBS, the smears were incubated with anti-mouse IgM (μ-chain specific) fluorescein isothiocyanate (FITC) conjugate (Sigma, Prague, Czech Republic), diluted 1:128 in PBS and incubated for 60 min at 37 °C, washed with PBS and distilled water and mounted in Vectashield H-1200 DAPI (Vector Laboratories Inc., Burlingame, CA). The Annexin V-FITC apoptosis detection kit (Sigma, Prague, Czech Republic) was applied for detection of the sperm damage according to laboratory instructions. Samples were examined with a Nikon Eclipse E400 fluorescent microscope equipped with a Nikon Plan Apo VC 60/1.40 oil objective and photographed with a CCD 1300-VDS camera (Vosskühler GmbH, Osnabrück, Germany) with the aid of the NIS-ELEMENTS Ar imaging software (Laboratory Imaging Ltd., Prague, Czech Republic).

2.6. Histological analysis and tissue morphometry

The right testis was fixed in 4% formaldehyde in PBS. The standard paraffin-embedded 2–3 μm thick tissue sections were prepared and stained by haematoxylin–eosin staining. Tissue specimens were evaluated under a light microscope. In all specimens, 100 seminiferous tubules were analyzed by computer-assisted morphometry. The thickness of the germinal epithelium and diameter of the seminiferous tubules were measured. The results were statistically evaluated.

2.7. TUNEL analysis

The number of apoptotic cells in tissue sections of the control and experimental animals was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), using an in situ detection kit (Promega, Madison, USA) according to the manufacturer's instruction. In brief, a paraffin-embedded tissue section was rehydrated in water, fixed in 4% formaldehyde, incubated in proteinase K solution (20 μg/ml) for 5 min, washed 2× in PBS, incubated for 10 min in equilibration buffer and finally exposed for 60 min to the labelling buffer containing both FITC-labelled dUTP and terminal deoxynucleotidyl transferase (control samples without terminal deoxynucleotidyl transferase or treated with DNase were also prepared). Before examination, TUNEL-labelled samples were washed in SCC (saline-sodium citrate buffer) and in water, mounted by Vectashield with DAPI dye to visualize the nuclei. Specimens were evaluated under a fluorescent microscope. In all specimens, the number of TUNEL positive cells in 20 cross-sectioned seminiferous tubules was

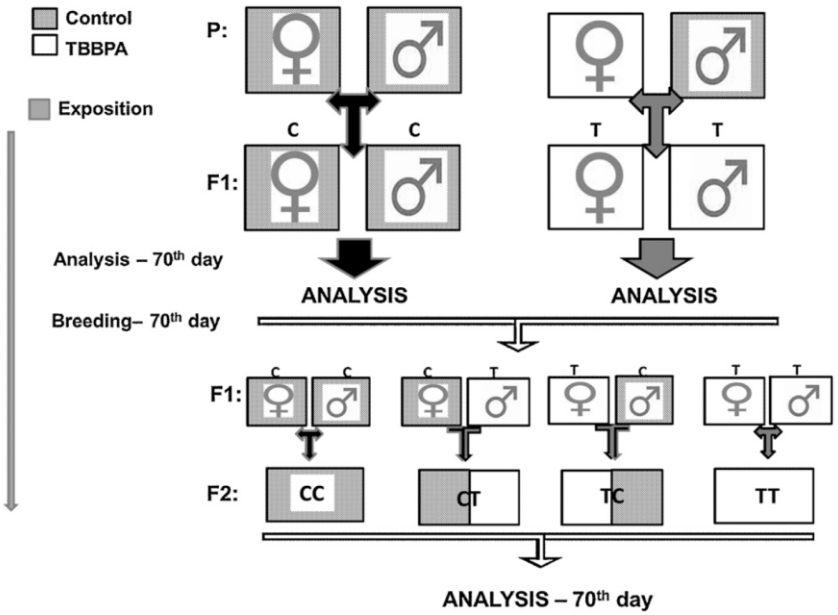

Fig. 1. Breeding diagram. Animals in parental generation were bred to form the F1 generation consisting of two groups (**C**; **T**). In group **C**, born pups were not affected by TBBPA. In group **T**, the pups were exposed to TBBPA in utero and postnatally. Animals of the F1 generation were bred to the age of 70 days as follows – mother and father from the group C formed group **CC** of the F2 generation; both parents from group T formed group **TT** (interbreeding), mother from group C and father from group T by outcrossing formed group **CT** and mother from group T and father from group C formed by reverse outcrossing group **TC** Pups born in the F2 generation were exposed to TBBPA only in groups TC and TT.

counted. Differences between the number of TUNEL-positive cells in the control and experimental samples were statistically analyzed.

2.8. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

First, total RNA was extracted from the testicular tissue. A Tri-Reagent kit (Sigma, Prague, Czech Republic) was used for RNA isolation. RNA was isolated from the left testes of CD1 mice, 1 ml of Tri Reagent was added, and the samples were then processed according to manufacturer's instructions. Isolated RNA was stored at −70 °C. The RNA quality and purity was measured spectrophotometrically in a spectrophotometer Helios α (Thermo Electron Corporation, Marietta, USA). The synthesis of cDNA was done using 5 μg of purified RNA with addition of 1 μl DNase I (Invitrogen, Carlsbad, USA), 1 μl DNase I reaction buffer (Fermentas, Burlington, Canada) and H_2O to reach a volume of 10 μl. This mixture was incubated for 30 min at 37 °C in a Touchgene Gradient Thermal Cycler (Techne, Burlington, USA). After incubation, 1 μl EDTA (Fermentas) was added and the mixture was further incubated at 65 °C for 10 min. 30 μl of the reaction mixture (8 μl of reaction buffer for M-MuLV reverse transcriptase (Fermentas, Burlington, Canada), 5 μl 10 mM 4dNTP (Fermentas, Burlington, Canada), 0.3 μl RiboLock inhibitor (Fermentas, Burlington, Canada), 1 μl oligo (dT) + random primers (Promega, Madison, USA) and 15.2 μl H_2O) was then added to the samples. The mixture was incubated for 60 min at 42 °C followed by 10 min at 70 °C and in the end was maintained at 4 °C. Obtained cDNA was stored at −20 °C. For RT-qPCR – 5× diluted cDNA was used. Used primers are summarized in Table 1. RT-qPCR reaction was carried out in PCR strips (BioRad, Prague, Czech Republic) and all work was performed in a sterile PCR box (Biosan, Riga, Latvia). For each reaction 2 μl 5× diluted cDNA, 10 μl SYBR Green Master Mix (Fermentas), 0.5 μl primer and 7 μl H_2O was used. All reactions were performed in duplets in a PCR cycler (Eppendorf, Prague, Czech Republic). The relative amount of mRNA in each sample was calculated from the measured CT values. The control was set at 100% and experimental samples were compared to the control (samples from the F1 generation were compared to the group C and samples from the F2 generation were compared to the group CC). The expression of the gene for peptidylprolyl isomerase A (PPIA) was used to normalize the measured values. The tested genes and primer sequences are summarized in Table 1. Number of analyzed animals in each group was 12 and for each group were performed 12 RT/qPCR reaction.

2.9. Statistical analysis

Statistical analysis was performed in STATISTICA 7.0 (StatSoft, Prague, Czech Republic). The number of offspring in the first generation was analyzed by the Mann–Whitney *U*-test. For analysis of the second generation the Kruskal–Wallis ANOVA test was used. Post hoc analysis in the second generation was done by using MCMR. The weight of the body and organs was compared using the analysis of variance (ANOVA), post hoc analysis was performed using the Newman–Keuls test. The weights of individual organs were compared using the analysis of covariance (ANCOVA) and the body weight was used as a covariate. Data obtained from sperm analysis and TUNEL method was analyzed using the Kruskal–Wallis ANOVA; post hoc analysis was performed again using MCMR. Differences in gene expression were analyzed by ANOVA; post hoc analysis was performed using the Newman–Keuls test. A *P*-value lower than 0.05 was identified as statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Number of progeny; body and organ weight

During the developmental period male mice were exposed to TBBPA (during gestation, lactation, pre-pubertal and pubertal period, and up to adulthood). To evaluate the effect of TBBPA on the male reproductive tract the body and organ weight, number and sex ratio of progeny and anogenital distance (AGD) were measured. Individuals from group T (see Section 2.1) were compared with the control animals from group C – F1 generation. Animals from the experimental groups of the F2 generation (CT, TC, and TT) were also compared with the control animals (CC) from the F2 generation. This procedure was used in all subsequent measurements.

TBBPA has no effect on the number of progeny and sex ratio in both generations. AGD and body weight were not affected in both generations as well. In the group TT of F2 generation, significantly reduced testicular weight was observed. In this group, increased weight of the prostate and seminal vesicles was also noticed. The weight of epididymis in animals from group CT of the F2 generation was also significantly increased. All measured values are summarized in Table 2.

Table 1
Primer sequences.

Genes	Accession no.	Nucleotide sequence	Size of PCR product (bp)
Acr	NC_000081.5	5′-cacgtgtggacctcattgac-3′ 5′-gtagtcccaggtggctgtgt-3′	248
Ar	NM_013476.3	5′-ggaccatgttttacccatcg-3′ 5′-tcgtttctgctggcacatag-3′	171
Apg-1	NM_011020.3	5′-gtcagaccttcccctgaaca-3′ 5′-gctccttgactgcaggaatc-3′	215
Hsp60	NM_010477.4	5′-cttcaggggttgtcacaggt-3′ 5′-atctattgccaaggagggct-3′	137
Hsc70t	NM_013558.2	5′-cctgaccaaggaggagattg-3′ 5′-tccttcagaccctcatcacc-3′	153
Hsp70-2	NM_008301.4	5′-gcgcctcacccaactagata-3′ 5′-gatctccaccttgccatgtt-3′	145
Bax	NC_000073.5	5′-caacttcaactggggccgcg-3′ 5′-tggatccagacaagcagccgc-3′	150
Bcl-2	NC_000067.5	5′-cagggatgtcacccctggtgg-3′ 5′-aggcatcccagcctccgttatcc-3′	104
Sox9	NM_011448.4	5′-gctggaagtcggagagccgaga-3′ 5′-agagaacgaaaccggggccac-3′	147
PPIA	NC_000077.5	5′-agctctgagcactggagaga-3′ 5′-gccaggacctgtatgcttta-3′	158

Table 2
Effect of TBBPA on body and organ parameters in CD1 mice. Number of analyzed animals in each group (n) was 20.

Groups	n	AGD (cm)	Body weight (g)	Testes (mg)	Prostate (mg)	Seminal vesicles (mg)	Epididymis (mg)
C	20	1.32 ± 0.18	29.39 ± 3.11	216.65 ± 47.01	101.65 ± 23.65	194.76 ± 34.78	72.41 ± 12.15
T	20	1.26 ± 0.13	31.44 ± 2.77	224.53 ± 23.59	112.53 ± 22.74	208.93 ± 40.90	76.73 ± 9.02
CC	20	1.39 ± 0.18	31.32 ± 1.54	223.25 ± 26.86	110.88 ± 25.05	193.19 ± 36.61	70.38 ± 5.82
CT	20	1.29 ± 0.18	30.22 ± 3.58	218.27 ± 29.72	113.47 ± 30.59	204.27 ± 42.04	**73.93 ± 8.21****
TC	20	1.32 ± 0.08	30.71 ± 1.81	221.33 ± 17.94	106.00 ± 42.78	216.44 ± 17.68	72.56 ± 7.37
TT	20	1.37 ± 0.18	31.47 ± 3.88	**203.27 ± 28.24***	**127.13 ± 24.28***	**232.25 ± 33.79****	72.13 ± 7.32

Mean ± SD.
* $P < 0.05$.
** $P < 0.01$.

Table 3
Effect of TBBPA on sperm parameters of CD1 mice treated with TBBPA. The sperm state was analyzed by monoclonal antibodies against intra-acrosomal sperm proteins (pHs-14; pHs-8), Hoechst 33342 and Annexin V kits. The mixture of sperm suspension was smeared onto glass slides for immunocytochemical analysis. Two hundred cells (sperm) were evaluated on every glass slide; number of analyzed animals in each group (n) was 20.

Groups	n	Sperm morphology (% of normal cells)	Sperm viability (% of live cells)	Annexin V (% of positive cells)	HS-14 (% of acrosome positive cells)	HS-8 (% of acrosome positive cells)
C	20	92.88 ± 2.74	90.24 ± 3.01	51.94 ± 10.28	61.53 ± 7.65	57.18 ± 5.43
T	20	92.70 ± 5.09	89.13 ± 2.36	49.67 ± 11.08	63.13 ± 7.77	59.47 ± 7.03
CC	20	89.87 ± 2.24	89.68 ± 1.77	51.06 ± 9.01	61.87 ± 2.41	57.18 ± 1.68
CT	20	89.06 ± 2.77	89.69 ± 2.33	50.13 ± 14.15	61.81 ± 7.52	55.81 ± 3.15
TC	20	88.44 ± 3.13	89.89 ± 1.17	59.89 ± 10.56	64.56 ± 2.40	55.00 ± 3.04
TT	20	90.27 ± 1.98	90.33 ± 1.91	43.67 ± 9.80	52.60 ± 4.07	55.00 ± 3.00

Mean ± SD.

3.2. Sperm parameters

To assess the effect of TBBPA on sperm quality, the sperm morphology, viability and state of acrosome were evaluated. In all groups about 90% of morphologically normal sperm was observed, and the differences between groups were minimal. A similar pattern was observed when evaluating the sperm viability. The apoptotic stage of spermatozoa was assessed by Annexin V. In all groups, similar numbers of Annexin-positive cells were obtained by measurements. In animals from group TC of F2 generation, a slightly increased number of Annexin-positive cells was noticed, but this value did not reach significant levels. The state of the acrosome was tested with monoclonal antibodies HS-8 and HS-14. Acrosome staining was comparable in all groups of both generations. Data are summarized in Table 3.

3.3. Histology of testes

Histological analysis was performed using testis paraffin sections. Morphology of seminiferous tubules and the process of spermatogenesis were evaluated visually under the microscope. In experimental groups no pathological patterns in morphology of seminiferous tubules were observed compared to the control. Also, the process of spermatogenesis was not interrupted. For all groups in both generations, spermatogonia, spermatocytes and prolonged spermatocytes released into the lumen of seminiferous tubules were visible (Fig. 2).

However, morphometrical analysis revealed changes in other histological parameters. Epithelial thickness and tubule diameter were analyzed. Whereas the diameter of the seminiferous tubule did not show any significant changes, the seminiferous epithelium

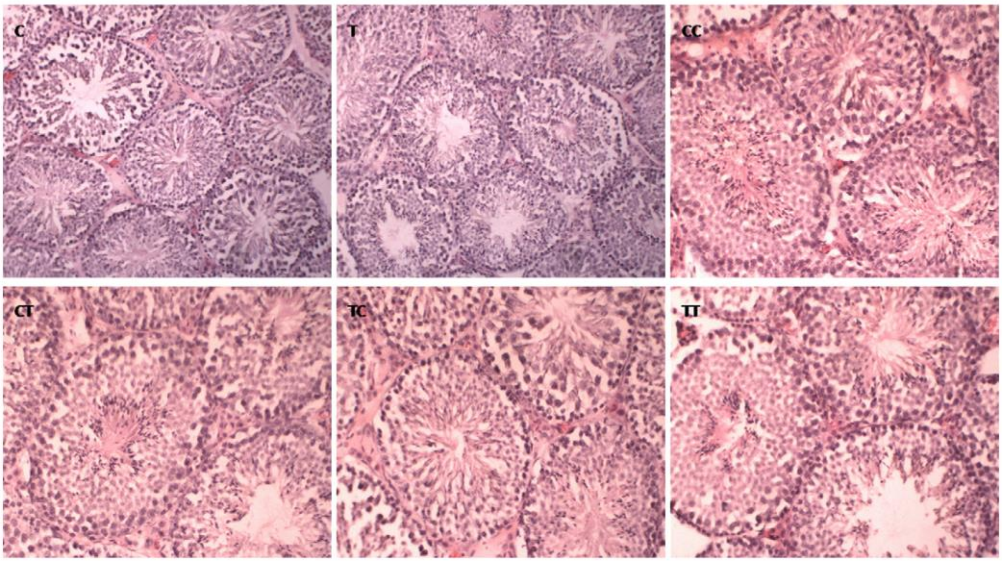

Fig. 2. Histological analysis of the testes from control and experimental animals. Paraffin sections were placed onto glass slides and stained by eosin (cytoplasm) and haematoxylin Harris (nucleus). Histological samples were analyzed under the microscope. Normal morphology and the process of spermatogenesis were observed in all experimental groups and compared to the control groups. Magnification 600×.

Table 4
Morphometrical and TUNEL analysis of seminiferous tubules. Testis paraffin sections were placed onto glass slides, where epithelial thickness as well as tubule diameters were measured and TUNEL analysis was performed. In all specimens, the thickness of the germinal epithelium and diameter of the 100 seminiferous tubules were analyzed and the number of TUNEL positive cells in 20 cross-sectioned seminiferous tubules was counted; number of analyzed animals in each group (n) was 20.

Groups	n	Epithelial thickness (μm)	Tubule diameter (μm)	Number of TUNEL positive cells
C	20	61.8 ± 3.61	225.5 ± 12.3	25.65 ± 1.62
T	20	**57.3 ± 4.61***	214.4 ± 11.5	**42.27 ± 6.10****
CC	20	59.5 ± 3.05	215.6 ± 17.4	24.67 ± 1.37
CT	20	58.0 ± 3.56	220.1 ± 23.7	26.00 ± 3.22
TC	20	**56.1 ± 3.13***	219.7 ± 23.44	**31.00 ± 2.10****
TT	20	**56.4 ± 3.22***	218.1 ± 21.3	**30.50 ± 2.81****

Mean ± SD.
* $P < 0.05$.
** $P < 0.01$.

was significantly lower in groups T (F1 generation) and TC; TT (F2 generation). Results are summarized in Table 4.

3.4. TUNEL analysis

To determine the number of apoptotic cells in the testes the TUNEL analysis of testis paraffin sections was performed. The highest increase of apoptotic cells in the testes was observed in group T of the F1 generation, where the number of apoptotic cells was almost two times higher in comparison to the control group. The increased number of apoptotic cells was also observed in groups TT and TC of the F2 generation, where about 25% more TUNEL-positive cells was measured compared to the control group (Table 4).

3.5. Real time PCR of testicular genes

Genetic analysis was performed by the quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) with primers for the genes of acrosomal proteins, androgen-responsive genes, heat shock protein (Hsp) genes, genes encoding proteins responsible for the regulation of apoptosis and a Sertoli cell-specific gene. The acrosome-specific gene tested was proacrosin (Acr). The androgen receptor (Ar) gene was selected as a member of the androgen-responsive genes. Genes tested for heat shock proteins were Hsp70-2, Hsc70t, Hsp60, and APG-1. The genes selected for their relation to apoptosis were Bcl-2 and Bax and the Sertoli cell-specific gene tested was Sox9 (Table 5). The Acr gene expression was comparable in all groups in both generations. No significant changes of gene expression for the androgen receptor were observed among the males of F1 generation. Contrary to that, the groups TC and TT had significantly reduced ($P < 0.001$) expression of this gene by about 20% compared with the control.

Our experiments showed relatively large changes in the expression of selected heat shock protein-encoding genes, with the exception of heat shock protein APG-1. The level of gene expression of APG-1 did not show any significant changes between the experimental and control groups. In contrast, a significantly reduced expression of the gene for Hsp70-2 in both generations ($P < 0.001$) was observed. The most reduced expression of this gene was observed in groups T of the F1 generation and TT of the F2 generation, reaching half the values compared with the control groups. A comparable pattern was observed for the Hsp60 gene, where the expression was significantly reduced in all experimental groups, both in F1 and in F2 generations ($P < 0.001$). Reduced expression was most apparent in the group T of the F1 generation and groups TC and TT of the F2 generation, where it reached nearly half the

Table 5
Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) analysis of testicular acrosome-, androgen-, heat shock- and apoptosis-related genes. In this study, genes for proacrosin (Acr), androgen receptor (Ar), heat shock proteins – Apg-1, Hsp70-2, Hsp60 and Hsc70t, apoptosis-related genes such as Bax and Bcl-2 and Sertoli cell-specific gene Sox9 were tested. The table illustrates relative expression of the selected genes. Control groups represent 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups (T, CT, TC, TT) and the control groups (C, CC). Number of analyzed animals in each group was 12 (*nb*) and for each group were performed 12 RT/qPCR reaction (*nt*).

Groups	*nb/nt*	Acr (%)	Ar (%)	APG-1 (%)	Hsp70-2 (%)	Hsp60 (%)	Hsc70t (%)	Bax (%)	Bcl-2 (%)	Sox9 (%)
C	12/12	100.0 ± 11.3	100.0 ± 6.6	100.0 ± 7.2	100.0 ± 8.11	100.0 ± 8.2	100.0 ± 12.6	100.0 ± 11.5	100.0 ± 12.2	100.0 ± 10.6
T	12/12	96.3 ± 12.5	110.0 ± 6.2	85.3 ± 12.6	**54.2 ± 7.4****	**60.7 ± 6.9****	**142.7 ± 13.5****	**144.7 ± 10.6****	**60.3 ± 9.1****	**45.5 ± 7.5****
CC	12/12	100.0 ± 11.5	100.0 ± 6.4	100.0 ± 7.9	100.0 ± 9.8	100.0 ± 11.2	100.0 ± 12.4	100.0 ± 11.5	100.0 ± 11.1	100.0 ± 20.3
CT	12/12	96.3 ± 11.7	94.8 ± 5.1	107.8 ± 13.9	**79.2 ± 8.4****	**72 ± 6.4****	102.0 ± 14.2	108.5 ± 13.3	91.0 ± 12.2	93.1 ± 15.11
TC	12/12	**108.8 ± 11.7**	**74.3 ± 7.9****	**116.8 ± 10.3**	**59.7 ± 8.9****	**59.8 ± 10.8****	**140.5 ± 7.6****	**128.8 ± 8.1****	**82.5 ± 9.1***	**53.6 ± 13.1****
TT	12/12	89.3 ± 10.2	**85.7 ± 6.9****	91.5 ± 6.4	**51.5 ± 8.1****	**56.6 ± 10.8****	**145.8 ± 14.8****	**134.8 ± 9.5****	**71.8 ± 9.4****	**38.6 ± 3.2****

Mean ± SD.
* $P < 0.05$.
** $P < 0.01$.

values compared with the control group. The expression level for Hsc70t was higher in group T of the F1 generation and groups TC, TT of the F2 generation ($P < 0.001$). The highest increase in expression was observed in groups T (F1 generation) and TT (F2 generation), where they reached almost 50% higher values than they did in the control group.

A differential expression pattern was also observed in genes encoding apoptotic genes Bax and Bcl-2. The most increased gene expression of Bax was observed in group T (F1 generation) and groups TC, TT (F2 generation), where 40% and 30% higher expression compared with the control group was detected. In contrast, low levels of expression were observed in the case of anti-apoptotic gene Bcl-2. Significantly reduced expression was detected in group T (F1 generation) ($P < 0.001$) and groups TC ($P < 0.05$), TT ($P < 0.001$) (F2 generation). The highest decrease in gene expression was observed in group T of the F1 generation, where the expression level was reduced by 40% compared with the control group.

The expression of Sertoli cell-specific gene Sox 9 was significantly decreased in the experimental groups, particularly in group T of F1 generation ($P < 0.001$) and groups TC and TT of F2 generation ($P < 0.001$) (Table 5).

4. Discussion

In this study we tested the effect of TBBPA on the reproduction parameters of CD1 mice. We nalyzed the following parameters: the numbers of progeny, body and organ weight, anogenital distance, sperm quality, histopathology of testes, apoptotic stage of testicular cells and activity of selected genes (acrosomal, androgen-responsive, heat shock and apoptotic genes). For our experiment, we used the CD1 outbred mice strain, because it simulates the human population more closely than inbred strains given its high heterozygosity. Experimental mice were exposed to the long-term effects of TBBPA. The substance was administered dissolved in water at a concentration of 200 μg/l.

Previous studies have shown that TBBPA can affect the thyroid hormonal system, mainly by binding to thyroid hormone transport protein – transthyretin [24–26]. Also the oestrogenic hormone system can be potentially affected by TBBPA. It was demonstrated that TBBPA is capable of binding the oestrogen receptor in vitro [28]. TBBPA can also induce apoptosis of TM4 Sertoli cells in vitro [34], but little is known about its effect on the reproductive parameters in vivo.

Our histological analysis showed no visible abnormalities or pathological changes in the morphology of seminiferous tubules of experimental animals (Fig. 1.). However, TUNEL analysis of histological sections of the testes showed a significantly increased number of apoptotic cells in the testes of experimental animals in groups T (F1 generation) and TC, TT (F2 generation) (Table 4). Mice in these groups also displayed significantly increased expression of the gene for pro-apoptotic protein Bax and decreased expression of the gene for anti-apoptotic protein Bcl-2 (Table 5). Proteins Bax and Bcl-2 play a key role in the activation of cell apoptosis. Anti-apototic protein Bcl-2 is located on the outer mitochondrial membrane and binds to the pro-apoptotic protein Bax. If this bond is not present, protein Bax becomes active and forms a homo-oligomeric channel, allowing an influx of ions into the mitochondria and thus initiating the activation of the apoptotic process. Therefore, unbalanced expression of these proteins could lead to the induction of apoptosis [35].

The groups in which an increased number of apoptotic cells was detected also had a significantly decreased expression of the Hsp60 gene (Table 5). Hsp60 is a mitochondrial protein that serves as a chaperone of the newly imported proteins and also helps transport proteins into the mitochondrial matrix and intra-membrane

space. The Hsp60 function is essential for the proper functioning of mitochondria [36,37].

Morphometrical analysis revealed significantly thinner seminiferous epithelium in groups T (F1 generation) and TC, TT (F2 generation) (Table 5). This may be a result of an increased incidence of apoptosis in the testis of mice in these groups. According to the study of Ogunbayo et al. [34], who showed that TBBPA is able to induce apoptosis in Sertoli cells, we assume that the apoptosis of Sertoli cells is the main cause of the decrease of thickness of the seminiferous epithelium. To support this hypothesis of TBBPA affecting Sertoli cells, we analyzed the expression of the nuclear transcription factor Sox9 gene. Sox9 is preferentially expressed in Sertoli cells and can serve as a marker of Sertoli cells. We detected decreased expression of the Sox 9 gene in groups T (F1 generation) and TC; TT (F2 generation), the same groups in which we detected the increased incidence of apoptosis in the testes.

Based on our results we can assume that the increase of apoptosis in the testes of groups T (F1 generation) and TC, TT (F2 generation) was caused by damage or malfunction of the mitochondria. This hypothesis is supported by the results of Ogunbayo et al. [34], who demonstrated that TBBPA causes dramatic changes in the mitochondrial membrane potential and subsequently the apoptosis of TM4 Sertoli cells in vitro. It is likely that changes in the mitochondrial membrane potential induce the release of various pro-apoptotic factors and subsequently induce programmed cell death in these cells in vitro [38].

In all experimental groups of F1 and F2 generations we observed decreased expression of the gene for Hsp70-2 (Table 5). Hsp70-2 is expressed during the meiotic phase of spermatogenesis, mainly in the pachytene spermatocytes [39]. It has been shown that the lack of expression of this gene causes disruption of spermatogenesis and induces apoptosis of spermatogenic meiotic cells [40]. Widlack et al. [41] showed that constitutive expression of active HSF1 leads to the induction of caspase-3-dependent apoptosis, and among others, down-regulation of Hsp70-2. HSF1 is the primary transcription factor responsible for the response to various forms of cellular stress and its constitutive expression in cells simulates exposure to permanent stress [42]. This indicates that TBBPA could cause stress conditions in spermatogenic cells that could lead to a decrease in the expression of Hsp70-2, which in turn could contribute to an increased number of apoptotic cells in the testes of experimental animals.

We also detected the expression of Hsc70t gene. Hsc70t is expressed specifically in the testes during the post-meiotic stages of spermatogenesis with the highest incidence in elongating spermatids [43]. It has been shown that the lack of this protein causes rapid loss of sperm motility [44], but its exact function is not yet known. In groups T (F1 generation) and TC, TT (F2 generation) we observed increased expression of this gene (Table 5). This again could be induced by stress, as a result of the exposure of experimental animals to TBBPA.

Another gene detected was proacrosin (Acr). Proacrosin is one of the main acrosomal proteins that becomes active during the acrosome reaction and binds to the glycoproteins of zona pellucida [45]. This gene was selected as a marker of acrosomal damage. The expression of this gene was comparable in all groups in both generations (Table 5). Data obtained from immunocytochemistry using specific antibodies against intra-acrosomal proteins (Hs-14 and Hs-8) did not reveal any damage to the acrosome (Table 3). It can therefore be assumed that TBBPA has no negative influence on the development and function of the sperm acrosome.

The expression of the gene for androgen receptor (Ar) in groups TC and TT (F2 generation) was reduced (Table 5). Ar is a nuclear receptor that becomes active after the binding of its ligand (natural ligands – testosterone and dihydrotestosterone). After its activation Ar is translocated to the nucleus where it functions as a transcriptional factor and activates transcription of androgen-dependent genes. The reduced expression of the Ar gene was also observed in CD1 mice affected by anti-androgen vinclozoline [2] or in rats affected by flutamide (anti-androgen drug administered to treat cancer) [46]. It can therefore be assumed that TBBPA could have a weak anti-androgen effect, which was apparent in the second generation of the experimental animals.

The groups most affected by TBBPA were T (F1 generation) and TT (F2 generation). In the second generation, the influence of TBBPA had a stronger effect than in the first generation. The reproductive organs of group TT displayed lower weight compared to the control and also decreased expression of the gene for androgen receptor up to the second generation.

Differing expression of the selected genes and increased apoptosis in the testes was detected in group TC (F2 generation). However, this group was affected less than group TT (F2 generation). We may thus assume that for the TBBPA activity it is important whether the parents were affected or not. This hypothesis is supported by the finding that changes in gene expression were observed even in group CT (F2 generation), where only the father was exposed to TBBPA (neither mother nor offspring were exposed to TBBPA). It thus seems that certain effects of TBBPA can be transmitted to the next generation. One possible route of such transfer may be epigenetic changes. It is hypothesized that exposure to environmental pollutants may induce epigenetic changes. These changes do not involve changes in the DNA sequence but may cause changes in gene expression and be transmitted to the next generations. This hypothesis was strongly supported by the study of Stouder and Paolini-Giacobino [47], who showed that endocrine disruptor vinclozoline is able to induce epigenetic changes. In their study administration of vinclozoline to pregnant female mice induced alterations in the imprinting of five paternally/maternally imprinted genes in the sperm of the offspring. Another study showed an association between imprinting errors and male infertility, specifically azoospermia [48]. Association between methylation defects and infertility itself was suggested in other studies as well [49–51]. These findings indicate that the possible mechanism of effect of environmental pollutants and the mechanism by which this effect is transmitted to the next generations could be via epigenetic changes. However, to be confirmed, this hypothesis needs further investigation.

5. Conclusion

In summary, our results provide evidence that TBBPA is capable of inducing apoptosis of testicular cells and changes in the morphometry of seminiferous tubules in CD1 mice. We assume that this involves also Sertoli cells, which provide support to developing spermatocytes. In this study, the investigated sperm parameters did not reveal any sperm damage. However, analysis of gene expression revealed changes in the expression of selected testicular genes. These genes were selected because of their essential role during spermatogenesis and because their impaired expression may negatively influence the course of this process. This two-generational in vivo study also suggests that permanent exposure to TBBPA slightly enhances its effect in the next generation depending on whether the parents were affected or not.

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Short Communication

Assessing Oestrogenic Effects of Brominated Flame Retardants Hexabromocyclododecane and Tetrabromobisphenol A on MCF-7 Cells

(endocrine disruptors / BFR / MCF-7 cells / *TFF1*)

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Abstract. Tetrabromobisphenol A (TBBPA) is the main flame retardant used in printed circuit boards and laminates. The human population is highly exposed to TBBPA as it is used in consumer electronics as well as office and communication equipment. The main use of hexabromocyclododecane (HBCD) is in insulation foam boards, which are widely used in the construction sector. Brominated flame retardants may possess endocrine disrupting activity and thus represent a threat to the environment, including humans and their reproduction. The aim of this work was to evaluate the oestrogenic effects of TBBPA and HBCD *in vitro* on MCF-7 cells. We used the proliferation test (E-screen assay) in MCF-7 breast cancer cells and reverse transcription quantitative polymerase chain reaction analysis of *TFF1* gene expression to analyse oestrogenicity of the studied compounds. RT-qPCR has proved to be a fast and valuable molecular technique in gene expression quantification. HBCD but not TBBPA increased cell proliferation in MCF-7 cells and up-regulated *TFF1* gene expression in a concentration-dependent manner. Anti-oestrogen ICI 182,780 inhibited up-regulation of *TFF1* by HBCD. We have shown that HBCD displays oestrogen-like effects on MCF-7 cells. TBBPA, on the other hand, has not shown any oestrogenic effect mediated by the oestrogen receptor α.

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Abbreviations: ATCC – American Type Culture Collection, BFR – brominated flame retardant, DC-FBS – dextran charcoal-stripped foetal bovine serum, DeBDE – decabromodiphenyl ether, DMEM – Dulbecco's Minimal Essential Medium, E1 – oestrone, E2 – 17-ß-oestradiol, E3 – oestriol, EE2 – 17-α-ethynyloestradiol, ERα – oestrogen receptor α, FBS – foetal bovine serum, HBCD – hexabromocyclododecane, MTT – 3-(4,5--dimethylthiazolyl-2) 2,5-diphenyltetrazolium bromide, PPIA – peptidyl-prolyl *cis-trans* isomerase A, RT-qPCR – reverse transcription quantitative polymerase chain reaction, SVHC – substance of very high concern, TBBPA – tetrabromobisphenol A, *TFF1* – trefoil factor 1.

Introduction

Brominated flame retardants (BFRs) have proved to be cost-effective and the most efficient flame retardants in plastics and textiles. With a wide use of plastic materials, there are growing concerns about the expansion of BFRs in the environment, their resistance and biodegradation. Due to bromide substituents, BFRs are considered to be toxic, persistent and bioaccumulative in the environment (Birnbaum and Staskal, 2004). BFRs at different concentrations have been measured in indoor and outdoor air and dust samples (Abdallah et al., 2008), in water, sediments, and in sewage sludge (de Wit, 2002). BFRs are detected in plants and wildlife throughout the food chain, in human tissues, blood serum, and in breast milk of the exposed occupational populations and in general population (for review see Jenssen et al., 2007; Lignell et al., 2009).

The highest-volume brominated flame retardant in use today is tetrabromobisphenol A (TBBPA) followed by decabromodiphenyl ether (DeBDE) and hexabromocyclododecane (HBCD). The primary use of TBBPA is as a reactive intermediate in the production of flame-retarded epoxy resins used in printed circuit boards. TBBPA belongs to the aromatic class of reactive BFRs, is chemically bound into plastic, and therefore only a minor part of TBBPA can leach out of the material. HBCD, a major brominated cycloaliphatic flame retardant, is primarily used in polystyrene foam and textiles (www.bsef.com). HBCD, which belongs to the cycloaliphatic class of the additive BFRs, is blended with polymers and hence can more easily diffuse into the environment. Despite the fact that HBCD is a very effective flame retardant and is thus used in lower concentra-

tions than other BFRs, it was found in samples in remote areas. Now it is considered to be persistent and bioaccumulative and was identified as a Substance of Very High Concern (SVHC) in the REACH programme list of the European Union in 2008 (www.echa.europa.eu).

In this study, we examined the oestrogenic effect of TBBPA and HBCD on human breast cancer cell line MCF-7. In addition to the analysis with widely used cell proliferation assay, we performed a very sensitive and fast protocol for the measurement of expression of the endogenous oestrogen-dependent *TFF1* (trefoil factor 1) gene.

Material and Methods

Chemicals

Oestrone (E1), 17-ß-oestradiol (E2), oestriol (E3), 17-α-ethynyloestradiol (EE2), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA) and fulvestrant (ICI 182,780) were all from Sigma-Aldrich (Prague, Czech Republic).

Cell culture

Human oestradiol-dependent breast cancer cell-line MCF-7 (American Type Culture Collection, ATCC) was maintained in Dulbecco's Minimal Essential Medium (DMEM, Sigma-Aldrich, Prague, Czech Republic), supplemented with 10% foetal bovine serum (FBS, Sigma-Aldrich, 1% penicillin-streptomycin (Sigma-Aldrich) in T-75 cm^2 flasks at 37 °C, in an atmosphere of 5% CO_2/95% air under saturating humidity. Stock culture was passaged every 3–4 days using a trypsin 0.25%-EDTA 0.02% solution (Sigma-Aldrich).

MTT proliferation assay

The cell proliferation rate was estimated by a modification of the MTT [3-(4,5-dimethylthiazolyl-2) 2.5--diphenyltetrazolium bromide] assay (Denizot and Lang, 1986). Briefly, cells were maintained in 24-well plates with oestrogen exhausted for five days in phenol red-free DMEM supplemented with 10% dextran charcoal-stripped FBS (DC-FBS, either commercial from Sigma-Aldrich or prepared in our laboratory according to Jørgensen et al. (2000)) before treatment. The cells were then exposed to the tested compounds for six days, the medium was exchanged every second day. For a negative control, cells were treated with 10 nM anti-oestrogen ICI 182,780. Then the cells were incubated in tetrazolium MTT (Sigma-Aldrich), dissolved at a final concentration of 1 mg/ml in serum-free, phenol-red-free medium, for a further 4 h. MTT-formazan was solubilized in isopropanol and the optical density was measured at a wavelength of 570 nm and a reference wavelength of 690 nm.

RNA isolation and RT-qPCR

Cells were plated in 24-well plates at a density of 5×10^4 cells/well. The day after plating, culture medium was changed to phenol red-free DMEM supplemented with 10% DC-FBS. Plastic ware was chosen carefully to minimize medium contamination with xenoestrogenic compounds (Ishikawa et al., 2001). Starting on day 5, after changing to oestrogen-free medium, the tested compounds were added at a range of concentrations and cells were treated for a total of 36 h (Jorgensen et al., 2000). Total RNA samples were isolated from cell cultures using an RNeasy mini-kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. The concentration and purity of the purified RNA was determined by UV spectrophotometry and confirmed by agarose gel electrophoresis. cDNA was synthesized from 1 μg of total RNA using SuperScript® III Reverse Transcriptase (Invitrogen, Prague, Czech Republic) or RevertAid™ Reverse Transcriptase (Fermentas, Burmington, Canada) and used for qPCR amplification of the *TFF1* gene using specific primers (forward: 5'-GAATTGTGGTTTTCCTGGTGTC-3';reverse:5'-AGCAGCCCTTATTTGCACACT-3') in a Mastercycler ep realplex real-time PCR system. The qPCR conditions were: initial denaturation 15 min, followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s. After PCR a melting curve was constructed by increasing the temperature from 72 to 95 °C to ensure that the correct product was amplified in the reaction. PCR was repeated three times in doublets for each gene, and the average Ct and standard deviations were calculated. *PPIA* was used as a reference gene and the primers were: forward: 5'-TTCATCTGCACTGCCAAGAC-3' reverse: 5'-TCGAGTTGTCCACAGTCAGC-3'.

Results and Discussion

In this study we investigated the oestrogenic activity of two brominated flame retardants: TBBPA and HBCD, comparing them with oestrogenic activity of known oestrogens and an anti-oestrogen.

MTT assay

To confirm the oestrogen responsiveness of the MCF-7 cell line, we started experiments with the MTT proliferation assay. Both E2 and EE2 induced cell growth at 10 pM concentration. Anti-oestrogen ICI 182,780 (10 nM) was used as a negative control and completely inhibited cell growth. TBBPA had no effect on cell growth even at a maximum concentration of 20 μM. On the other hand, HBCD at higher concentrations 10–20 μM induced cell growth in an oestrogen-dependent manner and ICI 182,780 inhibited the HBCD effect (Fig. 1).

TFF1 gene expression

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) has become an established and powerful technique for gene expression studies. Relative quantification is a crucial and frequently used method to assess RT-qPCR data, while target gene ex-

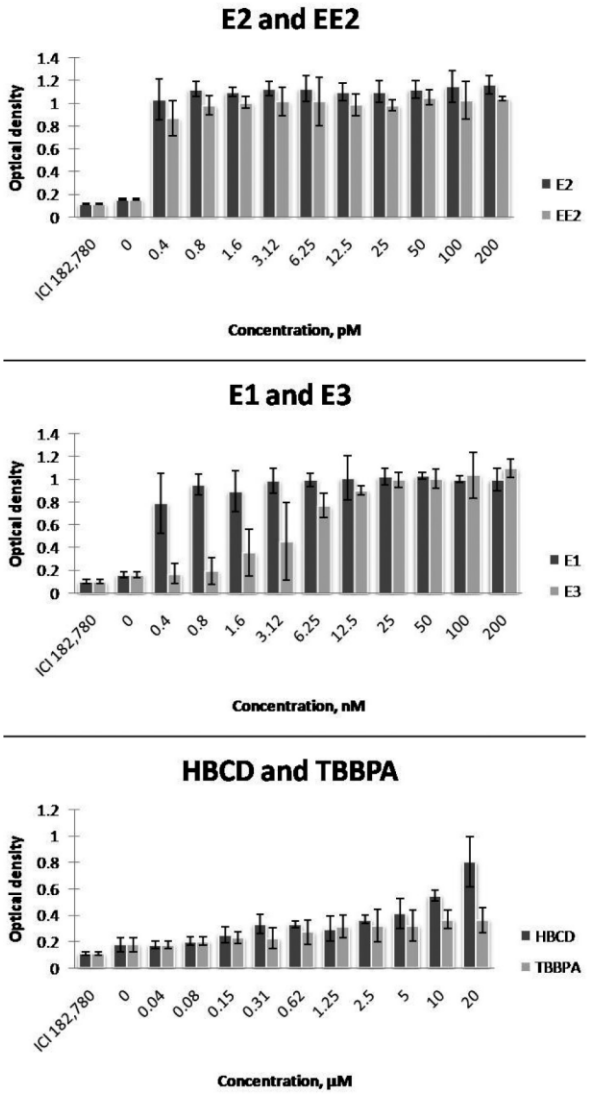

Fig. 1. Effect of E2, TBBPA and HBCD on MCF-7 cell proliferation. Cells were untreated (control), treated with 10 nM anti-oestrogen ICI 182,780, and treated with E2 in the concentration range 0.4 to 200 nM and with TBBPA and HBCD in the concentration range 40 nM to 20 µM. Numbers show optical density at 570 nm of solubilized MTT formazan.

pression levels are associated with a stably expressed internal reference gene determined in the same biological sample at the same time.

Up-regulation of the *TFF1* gene expression was used for the measurement of oestrogenicity because its expression is oestrogen receptor-mediated and its transcription is induced in MCF-7 cells by oestrogenic compounds. We detected an increase in the *TFF1* gene expression in MCF-7 cells for three natural oestrogens E1, E2 and E3 and one synthetic EE2. The highest levels of *TFF1* expression were observed at the concentrations of 20 pM and 200 pM for E2 and EE2, respectively. Both E1 and E3 have weaker oestrogenic activity and induced *TFF1* expression at the 1000-fold higher concentration of 200 nM. As expected based on the MTT proliferation assay, TBBPA had no effect on *TFF1* expression at the studied concentrations, whereas HBCD strongly up-regulated the expression of *TFF1* starting from 200 nM, reaching its peak at the concentration of 20 µM. In addition, ICI 182,780 (10 nM) abolished the oestrogenic effect of all studied compounds (Fig. 2).

TFF1, also known as *pS2*, is well recognized as a marker and control gene in the studies where the oestrogen-dependent gene expression is involved.

As TBBPA is produced at highest rates and HBCD is considered to be a persistent bioaccumulative, and as both of them are found ubiquitously in the environment, their potential effect on live organisms, including humans, has attracted a great deal of attention in recent years. The agonistic activity of TBBPA and HBCD on the thyroid hormonal activity has already been reported (Veldhoen et al., 2006; Saegusa et al., 2009; Sun et al., 2009). However, the data on their oestrogenic effect are still contradictory. It was shown that neither TBBPA nor HBCD display an oestrogenic effect on the vitellogenin serum level in rainbow trout (Ronisz et al., 2004). In another complex and systematic work on endocrine-disrupting potency of BFR, TBBPA and HBCD showed no oestrogenic activity in ER-CALUX assay (Hamers et al., 2006). On the other hand, TBBPA was shown to have weak affinity to the oestrogen receptor as well as a low estrogenic effect on MCF-7 cell proliferation, progesterone receptor and pS2 protein expression at higher concentrations of 30 µM (Olsen et al., 2003), but there is no further information on the potential oestrogenic effect of HBCD.

The structure of the ligand-binding site of the oestrogen receptors can precisely distinguish between oestrogens and androgens (Nahoum et al., 2003). However, due to its hydrophobic pocket nature, it often becomes the target for the synthetic endocrine disrupting compounds. Therefore, we studied whether HBCD and TBBPA can influence the oestrogen-responsive *TFF1* gene expression.

Our results show that TBBPA does not induce *TFF1* gene expression *in vitro.* On the other hand, HBCD induced both *TFF1* expression and MCF-7 cell proliferation at 20 and 200 nM concentrations. These concentrations are 10,000-fold higher than for natural oestrogen, E2, and at least 100-fold higher than the average concentration in the environment. Nevertheless, it shows that HBCD can influence live organisms by inducing expression of oestrogen-regulated genes. On the other hand, our results do not exclude the effect of TBBPA on live organisms. There are *in vivo* studies showing reproductive toxicity of TBBPA (Van der Ven et al., 2008), possibly by TBBPA binding to other nuclear receptors or by oestrogenic activity of its metabolites. In addition, it was also shown that hydroxylated products of TBBPA inhibit oestrogen sulphotransferase and can thus increase the oestrogen level in the organism (Kester et al., 2002).

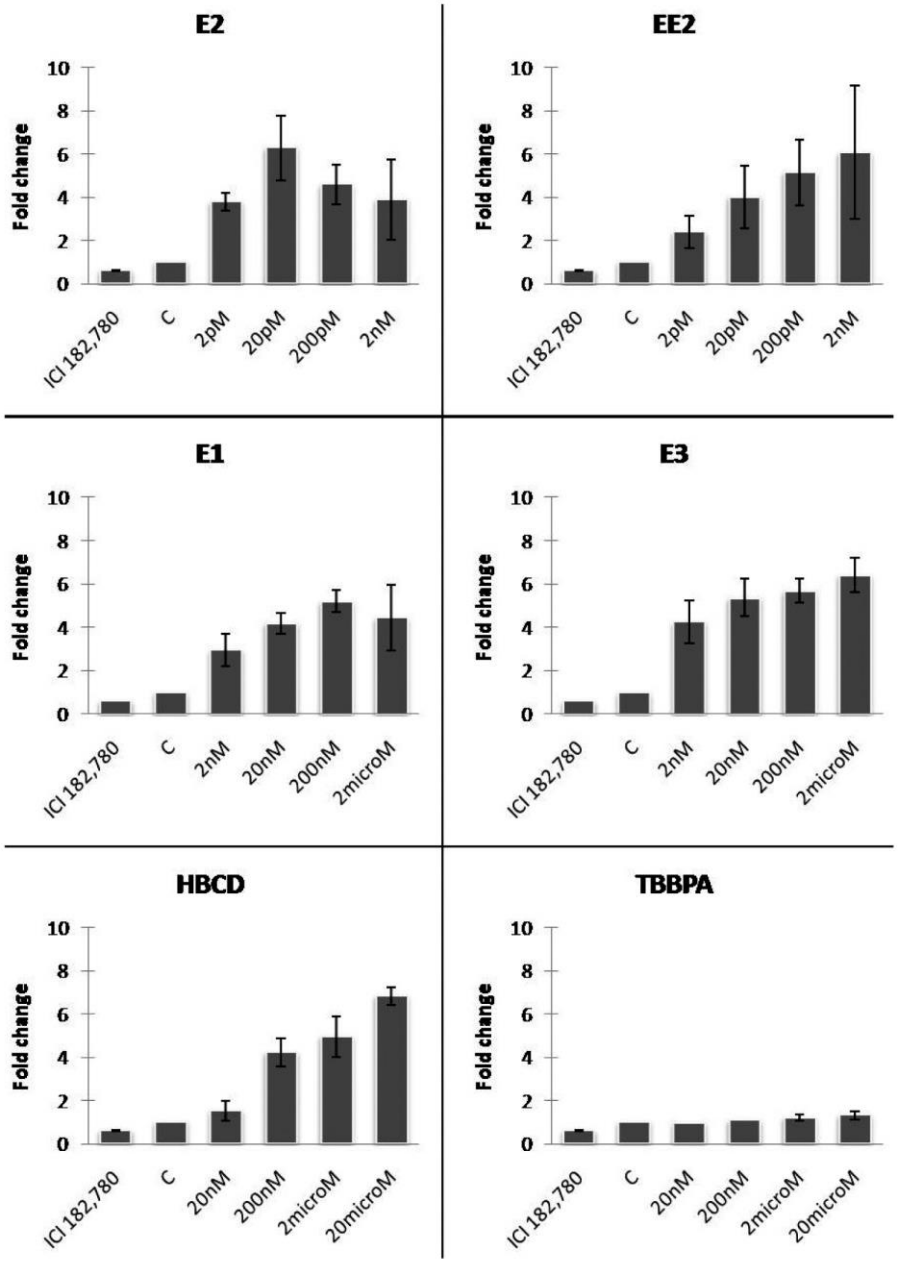

Fig. 2. Expression of the *TFF1* gene. Relative gene expression in untreated cells, cells treated with ICI 182,780 and a range of concentrations of the studied compounds. The values are the means of double determinations on each of the three replicate exposures, and are presented as the mean ± SEM.

To sum up, our current study demonstrated that *TFF1* gene expression data were in agreement with the MTT proliferation assay and that HBCD at higher concentrations induced both proliferation of MCF-7 cells and expression of the *TFF1* gene, thus displaying oestrogenic properties. Therefore, HBCD has the potential to disrupt the endocrine system. TBBPA, on the other hand, has not shown any oestrogenic effect at any measured concentration.

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The authors declare that they have no competing financial interests.

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8. List of publications

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1. **Margaryan H*, Dorosh A*, Capkova J, Manaskova-Postlerova P; Philimonenko A, Hozak P, Peknicova J.** Characterization and possible function of glyceraldehyde-3-phosphate dehydrogenase-spermatogenic protein GAPDHS in mammalian sperm. Reprod Biol Endocrinol. 2015 Mar 8;13:15

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2. **Zigo M, Dorosh A, Pohlová A, Jonáková V, Šulc M, Maňásková-Postlerová P.** Panel of monoclonal antibodies to sperm surface proteins as a tool for monitoring localization and identification of sperm-zona pellucida receptors. Cell Tissue Res. 2015 Mar;359(3):895-908

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3. **Zatecka E, Ded L, Elzeinova F, Kubatova A, Dorosh A, Margaryan H, Dostalova P, Korenkova V, Hoskova K, Peknicova J**. Effect of zearalenone on reproductive parameters and expression of selected testicular genes in mice. Reprod Toxicol. 2014 Jun;45:20-30

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4. **Dorosh A, Tepla O, Zatecka E, Ded L, Koci K, Peknicova J.** Expression analysis of MND1/GAJ, SPATA22, GAPDHS and ACR genes in testicular biopsies from non-obstructive azoospermia (NOA) patients. Reprod Biol Endocrinol. 2013 May 15;11:42

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5. **Elzeinová F, Pěknicová J, Děd L, Kubátová A, Margaryan H, Dorosh A, Makovický P, Rajmon R.** Adverse effect of tetracycline and doxycycline on testicular tissue and sperm parameters in CD1 outbred mice. Exp Toxicol Pathol. 2013 Sep;65(6):911-7

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Other publications

1. **Dorosh A.** Monoclonal antibody 6E4 against human GAPDHS protein. Hybridoma. June 2011, Vol. 30, No. 3: 321
2. **Ded L, Dostalova P, Dorosh A, Dvorakova-Hortova K, Peknicova J.** Effect of estrogens on boar sperm capacitation in vitro. Reprod Biol Endocrinol. 2010 Jul 13; 8:87.