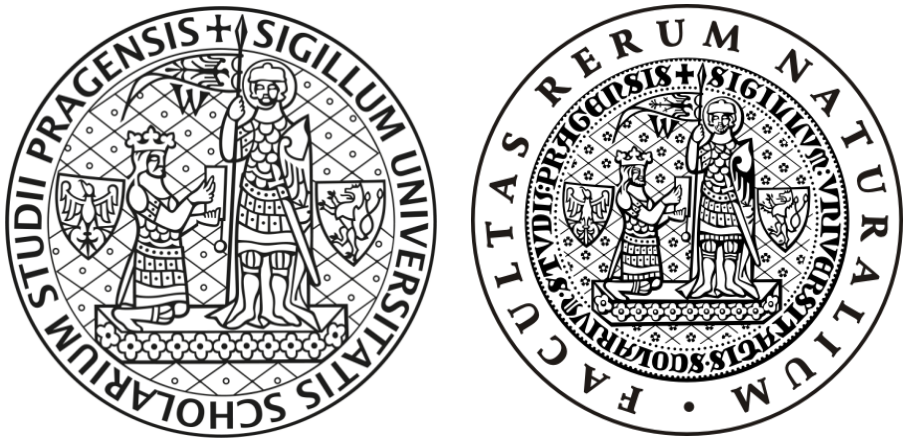
Charles University
Faculty of Science

Developmental and Cell Biology



MSc. Pavla Dostálová

The Influence of Estrogen and Endocrine Disruptors on Male Reproduction.

Vliv estrogenu a endokrinních disruptorů na samčí reprodukci.

Doctoral thesis

Supervisor:
doc. RNDr. Jana Pěknicová, CSc.

Prague, 2025

Statement:

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Prague, January 2025

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Abstract

Mammalian reproduction is a unique process that involves a series of precisely regulated events, including the production and maturation of gametes, fertilization, and both embryonic and postnatal development. The reproductive system is closely linked to the endocrine system, which regulates these physiological processes through hormones. A proper hormonal balance is crucial for successful reproduction. However, environmental substances known as endocrine disruptors (EDs) can mimic natural hormones and interfere with these processes.

Our study focused on reproduction from the male perspective. The first aspect we investigated was capacitation, the maturation process of sperm necessary for acquiring the ability to fertilize an egg. We found that elevated levels of natural estrogen, specifically 17β-estradiol, in males lead to premature capacitation of sperm in the tail of the epididymis, which can negatively affect the sperm's ability to reach the egg at the right time. This phenomenon may represent one of the ways in which endocrine disruptors with estrogenic activity contribute to the increasing prevalence of fertility issues.

Capacitation involves biochemical and molecular changes that can be monitored using various detection methods. Standardized assessment of capacitation is essential for accurately evaluating the fertilization potential of sperm exposed to physiological or environmental factors. In the next step, we compared four methods used—CTC, ACR.2, yP, and FITC-Phall—and confirmed that all effectively detect the progression of capacitation while highlighting their limitations and differences in detecting this process. Additionally, we described a timeline of molecular processes detected by these methods.

In the second part of presented work, we examined the effects of endocrine disruptors on male reproduction. Unlike toxicological studies, we tested the effects of EDs at significantly lower concentrations relevant to environmental exposure. All three tested EDs altered the expression profile of testicular cells. Using these three substances as examples, we demonstrated that exposure effects can be transmitted to subsequent generations; continuous exposure across generations can lead to cumulative negative effects (e.g., tetrabromobisphenol A). Interestingly, we found that a lower dose (relevant to human exposure) can have a greater impact than a higher dose (e.g., zearalenone). Finally, the study with di-(2-ethylhexyl) phthalate indicated that exposure to EDs during critical developmental periods, such as embryonic development and puberty, can affect reproductive health in adulthood.

Epidemiological data consistently indicate a significant decline in both sperm count and quality among men over recent decades. Evidence of the negative impact of EDs on male reproduction raises concerns about a potential link between increasing global environmental pollution and deteriorating sperm quality.

Souhrn

Reprodukce savců je jedinečný proces, který zahrnuje řadu přesně řízených událostí, včetně produkce a zrání gamet, oplození a embryonálního a postnatálního vývoje. Reprodukční systém je úzce spojen s endokrinním systémem, který reguluje tyto fyziologické procesy prostřednictvím hormonů. Správná hormonální rovnováha je zásadní pro úspěšnou reprodukci. Nicméně environmentální látky, známé jako endokrinní disruptory (EDs), mohou napodobovat přirozené hormony a narušovat fyziologické procesy.

Naše studie se zaměřila na reprodukci z pohledu samců. Prvním aspektem, který jsme zkoumali, byla kapacitace, maturační proces spermií nezbytný k získání schopností oplodnit vajíčko. Zjistili jsme, že zvýšená hladina přirozeného estrogenu, 17β-estradiolu, u samců vede k předčasné kapacitaci spermií v ocasu nadvarlete, což může negativně ovlivnit schopnost spermií dosáhnout vajíčka ve správný čas. Tento jev může představovat jeden ze způsobů, jak endokrinní disruptory s estrogenní aktivitou přispívají k rostoucí prevalenci problémů s plodností.

Během kapacitace dochází k řadě biochemických a molekulárních změn, které lze sledovat pomocí různých detekčních metod. Standardizované hodnocení kapacitace je nezbytné pro správné posouzení fertilizačního potenciálu spermií vystavených fyziologickým či environmentálním faktorům. V dalším kroku jsme porovnali čtyři používané metody hodnocení kapacitačního stavu spermií – CTC, ACR.2, yP a FITC-Phall. Ověřili jsme, že všechny testované metody efektivně detekují průběh kapacitace a zdůraznili jsme jejich limity a rozdíly v detekci tohoto procesu. Dále jsme popsali časovou osu molekulárních mechanismů detekovaných těmito metodami.

Ve druhé části práce jsme se zaměřili na vliv endokrinních disruptorů na samčí reprodukci. Na rozdíl od toxikologických studií jsme testovali vliv těchto EDs při výrazně nižších koncentracích relevantních pro environmentální expozici. Všechny tři testované EDs byly schopny změnit expresní profil testikulárních buněk. Na příkladu těchto tří látek jsme prokázali, že dopad expozice se může přenášet do následující generace, přičemž nepřetržitá expozice napříč generacemi vede k hromadění negativních účinků (tetrabrombisphenol A). Zajímavým zjištěním bylo, že nižší dávka (relevantní pro expozici člověka) může mít větší dopad na testované parametry než dávka vyšší (zearalenon). Konečně studie s di-(2-ethylhexyl) ftalátem ukázala, že expozice EDs během kritických vývojových období, jako je embryonální vývoj a puberta, může ovlivnit reprodukční zdraví v dospělosti.

Epidemiologické údaje konzistentně naznačují významný pokles jak počtu, tak kvality spermií u mužů během několika posledních desetiletí. Důkazy o negativním vlivu EDs na samčí reprodukci zvyšují možnost spojení nárůstu globálního znečištění životního prostředí se zhoršováním kvality spermatu.

Abbreviations

ACR.2	anti-acrosine antibody
AGD	anogenital distance
Apg-1	ATP and peptide-binding protein in germ cells-1
AR	acrosome-reacted
Ar	androgen receptor
ARE	androgen response element
Arpc1b	actin-related protein 2/3 complex subunit 1B
b.w.	body weight
Bax	bcl-2-like protein 4
Bcl2	B-cell lymphoma-2
BPA	bisphenol A
BTB	blood-testis barrier
C57BL/6J	inbred strain of laboratory mouse
Ca^{2+}	calcium ion
Ccna1	cyclin-A1
Ccnd1	cyclin D1
Cldn11	claudin-11
CTC	chlortetracycline
Ctsd	cathepsin D
DEHP	di-(2-ethylhexyl) phthalate
DNA	deoxyribonucleic acid
Dnmt1	DNA (cytosine-5)-methyltransferase
E2	17β-estradiol
ED(s)	endocrine disruptor(s)
Eea1	early endosome antigen 1
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
Eps8	epidermal growth factor receptor kinase substrate 8
ER(s)	estrogen receptor(s)

ERE	estrogen response element
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ES	ectoplasmic specialization
F1	first generation
F2	second generation
F3	third generation
F-actin	filamentous actin
FC	flow cytometry
FITC-phall	fluorescein isothiocyanate-conjugated phalloidin
Fkbp5	FK506 binding protein 5
FM	fluorescent microscopy
FSH	follicle-stimulating hormone
Fshr	follicle-stimulating hormone receptor
FVB/N	inbred strain of laboratory mouse
GH3 cells	cell line derived from pituitary gland
Gja1	gap junction alpha-1
GnRH	gonadotropin-releasing hormone
GPER	G protein-coupled estrogen receptor 1
Grth	gonadotropin-regulated testicular helicase
HCO_3^-	bicarbonate
HPG	hypothalamic-pituitary-gonadal axis
Hsc70t	heat shock cognate protein 70t
Hsp60	heat shock protein 60
Hsp70-2	heat shock protein 70-2
Icap1	integrin beta-1-binding protein 1
Igfbp5	insulin-like growth factor binding protein 5
Itgb1	integrin beta 1
Kdm4a	lysine-specific demethylase 4A
Lamc3	laminin subunit gamma-3

LC(s)	Leydig cell(s)
LH	luteinizing hormone
Lhcgr	luteinizing hormone/choriogonadotropin receptor
Mas1	mas proto-oncogene 1
MCF-7	cell line derived from human breast adenocarcinoma
MEHP	mono-(2-ethylhexyl) phthalate
Mit/E2	cell line
Mmp2	matrix metallopeptidase 2
Ncad	neural cadherin
NOAEL	No Observed Adverse Effect Level
Ocln	occludin
p21	cyclin-dependent kinase inhibitor 1A
p53	tumor protein p53
PND	postnatal day
Prm1	protamine 1
PTM	peritubular myoid cells
pY	phosphotyrosine
SC(s)	Sertoli cell(s)
Sox9	SRY-box transcription factor 9
Spata2	spermatogenesis-associated protein 2
Sycp1	synaptonemal complex protein 1
Sycp3	synaptonemal complex protein 3
TBBPA	tetrabromobisphenol A
TBC(s)	tubulobulbar complexe(s)
TDI	tolerable daily intake
Tes	testin
Tff1	trefoil factor 1
Timp2	tissue inhibitor of metalloproteinases 2
Tjp1	tight junction protein 1
TM4	Sertoli cell line

Tnp1	transition protein 1
Tnp2	transition protein 2
TTR	transthyretin
Vegfa	vascular endothelial growth factor A
Wt1	Wilms tumor 1
ZEA	zearalenone
ZP	*zona pellucida*

1 Introduction

1.1 Sperm production and maturation

In order to ensure the continued existence of species, living organisms have to reproduce themselves. Vertebrates, including humans, reproduce sexually, which requires a myriad of precisely orchestrated events, including gamete production and maturation, fertilization, and embryo development. During fertilization, male and female gametes fuse to give rise to a zygote, the single-cell embryo that is genetically unique and carries a combination of its parents´ genes. To ensure that the sexually produced offspring do not have double the number of chromosomes compared to the normal state, it is necessary to ensure that gametes are haploid. This is achieved by a unique cell division called meiosis that is part of gametogenesis.

1.1.1 Spermatogenesis

The male version of gametogenesis, spermatogenesis, takes place within the seminiferous tubules of the testes and gives rise to sperm. Spermatogenesis begins at the basal lamina of the seminiferous tubules, where the undifferentiated male germ cells, known as spermatogonial stem cells, are located. These cells undergo mitotic division, which gives rise to spermatogonia A, which replenish stem cells, and spermatogonia B, which differentiate into primary spermatocytes. Subsequently, the primary spermatocyte divides meiotically into two secondary spermatocytes (meiosis I), and each secondary spermatocytes divides into two haploid round spermatids (meiosis II). Round spermatids subsequently enter the process of spermiogenesis, in which they differentiate into elongated spermatids and finally into morphologically mature sperm [1] (Fig.1.). Spermiogenesis involves processes such as the condensation and shaping of the nucleus, acrosome (specialized Golgi derived organelle) and flagellum formation, and the formation and shedding of residual bodies. Sperm are then released into the lumen of the seminiferous tubule in a process called spermiation and are further transported to the epididymis [2]. Dividing germ cells do not complete cell division but remain connected by so-called cytoplasmic bridges, allowing for mutual communication [3].

As mentioned earlier, spermatogenesis occurs within the seminiferous tubules. However, these tubules are not only composed of germ cells at various develepmental stages but also contain indispensable somatic cells known as Sertoli cells (SCs). SCs establish and maintain

the spermatogonial stem cell niche and provide essential support to developing germ cells [4]. Furthermore, adjacent SCs create one of the tighest blood-tissue barrier, the blood-testis barrier (BTB), which separates the basal compartment of the seminiferous tubules from the adluminal compartment. As the BTB is semipermeable, it creates a unique environment for the sperm formation. The BTB also restricts contact between postmeiotic germ cells (in the adluminal part) and the immune system, thereby preventing the formation of autoimmune antibodies against germ cells that develop after immune tolerance is established [5]. The BTB is composed of tight junction proteins (e.g CLAUDINS, OCCLUDINS), gap junction proteins (e.g. GJA1), and adherens junction proteins. These junction proteins are cross-linked with the cytoskeleton via a scaffold protein [5].

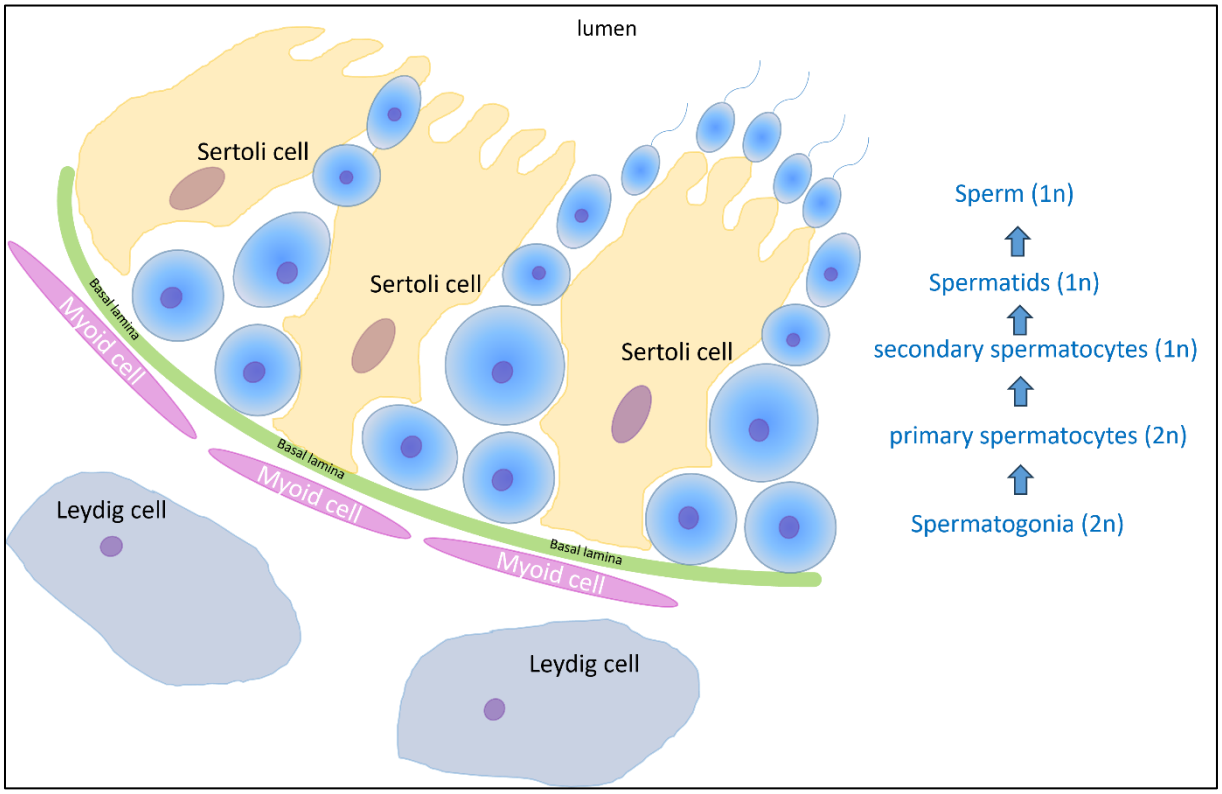


Fig.1. Seminiferous tubule. A cross section of the germinal epithelium in the seminiferous tubule with the description of individual cell types inside and outside the seminiferous tubule.

Germ cells are attached to Sertoli cells throughout spermatogenesis. However, during their development, germ cells migrate from the basal lamina through the BTB toward the lumen of the seminiferous tubules, where they are ultimately released. This process requires significant restructuring of unique anchoring junctions, such as ectoplasmic specializations (ES) and tubulobulbar complexes (TBCs), which are found exclusively in the testes [6] (Fig.2.).

The ectoplasmic specialization is an essential adherens junction that occurs between Sertoli cells (basal ES, restricted to the BTB) and between Sertoli cell and elongating spermatids (apical ES). The apical ES serves as the sole anchoring device for spermatids from step 8 onward and is composed of proteins such as NCAD, ITGB1, and LAMC3. Additionally, the proteins MMP2 and TIMP2 regulate the degeneration of the apical ES and subsequently remodeling of the BTB. These processes are necessary for spermiation and for the transit of preleptotene spermatocytes through the BTB, respectively. Interestingly, the transit of preleptotene spermatocytes across the BTB coincides with the timing of spermiation [7].

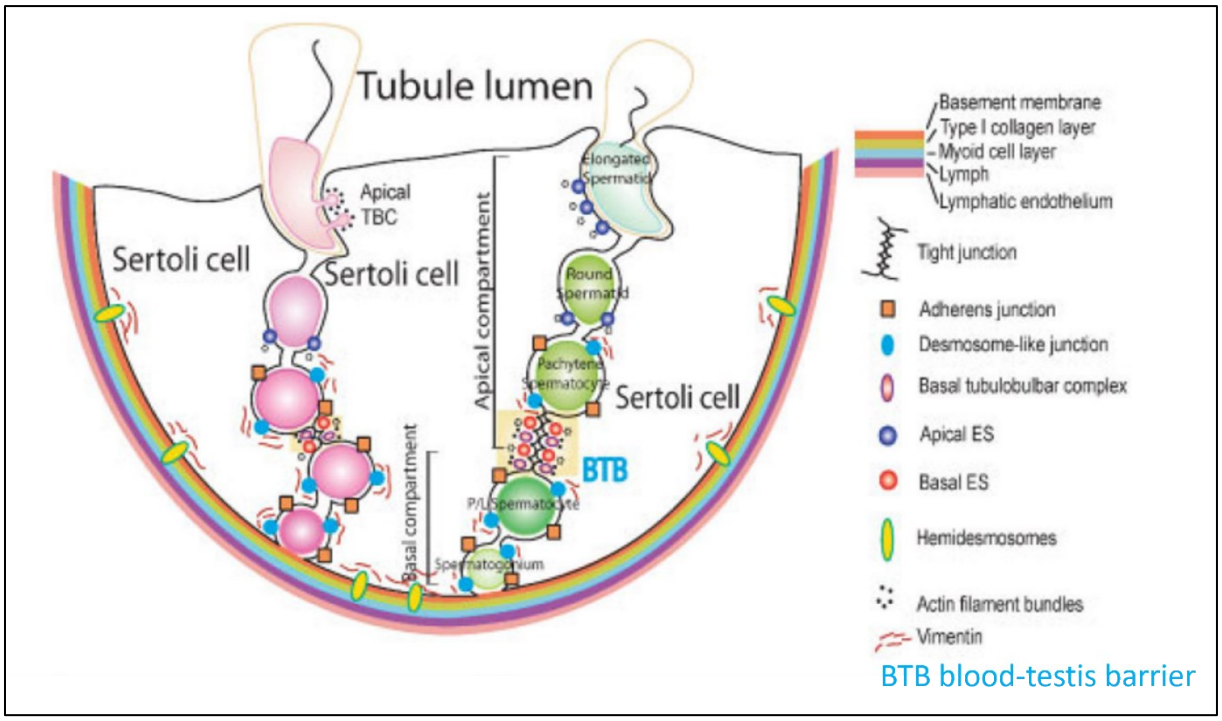


Fig.2. A schematic drawing to show the relative locations of junctions between Sertoli cells and Sertoli–germ cells [2].

Just prior to spermiation, apical ESs are partially replaced by apical TBCs on the concave surface of the spermatid head. TBCs thus form a transient anchoring device for mature spermatids before they are released. Furthermore, TBCs serve as endocytic devices that recycle junction proteins, play a role in eliminating excess spermatid cytoplasm, and contribute to the shaping of the spermatid acrosome [8].

As evident, interactions among germ cells, SCs, and germ-SC are essential for maintaining spermatogenesis. Any disturbance in these interactions may lead to damage in

spermatogenesis, reducing both the quantitative and qualitative output of spermatogenesis and ultimately affecting fertility.

The external wall of the seminiferous tubule is surrounded by peritubular myoid cells (PTM). These endothelial cells contract to force sperm down the tubule. In addition to maintaining the structure of the seminiferous tubule and facilitating the transport of spermatozoa and tubular fluid, PTMs produce and secrete several factors that act on SCs, thereby participating in the regulation of spermatogenesis [9].

Leydig cells (LCs) represent another testicular cell type that is necessary for the proper course of spermatogenesis. LCs are located in the interstitium, which is the space between the seminiferous tubules. They express steroidogenic genes and serve as the site of androgen (testosterone) synthesis. Testosterone acts as a paracrine factor that diffuses into the seminiferous tubules to regulate spermatogenesis [10]. The hormonal control of this process will be discussed further in the following section.

The testes are vital reproductive organs containing several unique cell types. They are also the place where unique processes such as meiosis, acrosome formation, flagellum development, and nuclear compaction occur. For the formation of morphologically mature sperm, it is crucial that all these processes take place correctly and at the appropriate times. This is achieved through regulated gene expression, where specific genes are expressed only at certain times and often exclusively by particular cell types. Monitoring the expression of specific genes can thus provide insights into specific processes or cell types involved in spermatogenesis.

1.1.2 Sperm maturation

Spermatogenesis is the initial step in sperm development. However, testicular sperm lack the necessary properties for fertilizing an egg. They acquire these properties during their subsequent maturation in both the male and female reproductive tracts. The first stage of this maturation occurs as sperm pass through the epididymis. After ejaculation, sperm undergo a series of maturation processes in the female genital tract, collectively called capacitation. Once capacitated, sperm can reach the egg, bind to the *zona pellucida* (ZP), undergo the acrosome reaction, and ultimately fertilize the egg.

1.1.2.1 Epididymal maturation

The epididymis is a tightly coiled tube that can be divided into three regions: the caput, corpus, and cauda. Sperm are transported from the testis through the caput and corpus to the cauda, where they are stored before being either ejaculated or degraded. During their passage through the epididymis, sperm undergo a process known as epididymal maturation, which renders them competent for capacitation and enables them to acquire progressive motility [11].

Epididymal maturation involves changes in the protein, sugar, and lipid content of sperm. Some proteins are lost, while others are incorporated or modified through processes such as phosphorylation and oxidation of thiol groups [11]. As highly differentiated cells, sperm are considered transcriptionally and translationally silent [12]. Therefore, newly incorporated proteins originate from the epididymis.

Changes in lipid composition lead to an increase in plasma membrane fluidity in sperm from the cauda compared to those from the caput. This increased fluidity plays a crucial role in enabling sperm to undergo the acrosome reaction and subsequently fuse with the egg [11].

1.1.2.2 Capacitation

As soon as sperm enter the upper female reproductive tract, they begin a comprehensive, multistep maturation process collectively called capacitation. Capacitation is an essential prerequisite for sperm to undergo the acrosome reaction, bind to and penetrate the *zona pellucida*, and ultimately fertilize the egg. This process was first described independently by Austin and Chang in 1951 [13, 14]. Since then, significant knowledge has been gained regarding capacitation; however, some aspects remain incompletely understood. Most current insights into capacitation derive from *in vitro* studies where epididymal or ejaculated sperm are incubated in a defined medium containing sources of Ca^{2+} and HCO_3^-, energy substrates, and cholesterol acceptors.

During capacitation, numerous changes occur in the sperm head. Sterols are released from the plasma membrane, resulting in increased membrane fluidity and permeability. This is followed by elevated intracellular concentrations of Ca^{2+} and HCO_3^-, which initiate a multi-step signalling cascade in which protein phosphorylation—primarily on tyrosine residues—plays a critical role. These signalling cascades lead to actin polymerization, creating a network between the plasma membrane and the outer acrosomal membrane. It has been shown that the breakdown of F-actin is necessary for the acrosome reaction to occur [15].

Further changes also take place in the sperm tail, resulting in a change in the pattern of sperm movement known as hyperactivation of motility. Hyperactivation facilitates sperm release from the oviductal reservoir and enables penetration through the cumulus oophorous surrounding the egg [15].

Given that capacitation is a complex biological process, several methods have been developed to detect different parameters associated with it. One of the most widely used methods is the chlortetracycline (CTC) assay. CTC detects calcium-associated membrane changes on the sperm head that occur during capacitation [16]. Three specific fluorescent patterns corresponding to sperm maturation status can be observed: 1. Non-capacitated, acrosome intact sperm - bright fluorescence over the entire sperm head and positive mid-piece of the tail; 2. Capacitated, acrosome-intact sperm - prominent fluorescence in the equatorial segment and mid-piece of the tail, with no fluorescence (dark) in the post-acrosomal region of the head; 3. Acrosome-reacted sperm - low fluorescence over the sperm head, with a remaining positive signal in the equatorial segment and mid-piece of the tail [16] (Fig.6., middle part).

Another method used in capacitation studies is the ACR.2 assay. The ACR.2 antibody recognizes and binds to acrosin epitopes. Acrosin is a digestive enzyme located inside the acrosome that aids sperm in penetrating the *zona pellucida*. During capacitation, changes occur in the accessibility of acrosin epitopes due to rearrangement of the acrosomal matrix. Increased accessibility of these epitopes corresponds to progress in capacitation; thus, non-capacitated sperm exhibit moderate fluorescence in the acrosomal region of the sperm head, while capacitated sperm show strong fluorescence. Acrosome-reacted sperm lose specific antibody binding capability, resulting in diminished fluorescence in this area [17, 18] (Fig.6., middle part).

Phosphorylation of several proteins is another parameter that can be monitored during sperm capacitation. Increases in protein phosphorylation can be detected using western blotting or various fluorescent techniques such as fluorescent microscopy, flow cytometry, or ELISA. For example, microscopic detection of fluorescently labelled phosphotyrosine provides three distinguishable patterns corresponding to non-capacitated, capacitated, and acrosome-reacted sperm [19] (Fig.6., middle part).

Finally, increases in actin polymerization during sperm capacitation represent another measurable parameter. For this purpose, fluorophore-conjugated phalloidin is commonly used. Although phalloidin is toxic to living cells, it can be employed to detect filamentous actin (F-

actin) in fixed and permeabilized cells. Detection with fluorophore-conjugated phalloidin allows for differentiation between non-capacitated, capacitated, and acrosome-reacted sperm [20] (Fig.6., middle part).

1.2 Endocrine system

Reproduction is regulated by the endocrine system, specifically the hypothalamic-pituitary-gonadal (HPG) axis (Fig.3.). During puberty, specialized neurons in the hypothalamus synthesize and release gonadotropin-releasing hormone (GnRH) in a pulsatile manner [21]. GnRH in turn stimulates the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland. These gonadotropins subsequently travel through the bloodstream to their target organs—in males, the testicles. FSH and LH bind to their respective receptors in Sertoli and Leydig cells, stimulating the production of inhibin B and testosterone, respectively. Both inhibin B and testosterone serve as feedback signals that regulate the HPG axis by downregulating gonadotropin secretion [22]. Testosterone can be further metabolized to estradiol by the enzyme aromatase in adipose tissue and within the testicles themselves [23].

FSH exerts its effects directly on Sertoli cells by regulating the proliferation and apoptosis of juvenile Sertoli cells during the fetal, neonatal, and peri-pubertal periods, as well as by influencing Sertoli cell differentiation during puberty [24]. It is generally accepted that Sertoli cell proliferation ceases during puberty [24], although some studies suggest that even adult Sertoli cells can proliferate [25, 26]. Since adult Sertoli cells can support only a limited number of germ cells, their quantity in adulthood influences spermatogenesis output [24]. FSH acts on germ cells indirectly by regulating factors produced by Sertoli cells. FSH signaling positively regulates the maintenance of the spermatogonia pool, initiates entry into meiosis, and limits overall germ cell apoptosis. Additionally, FSH influences the formation of ectoplasmic specializations [27].

As mentioned earlier, LH acts on Leydig cells to stimulate androgen (testosterone) synthesis. Testosterone in turn binds to androgen receptors (Ar) present on Sertoli cells, peritubular myoid cells, and Leydig cells [10]. The presence of Ar in germ cells remains a topic of debate [28]. Regardless, functional studies indicate that Ar is not required in germ cells [10]. Most effects of testosterone on spermatogenesis are mediated indirectly through its action on Sertoli cells. Testosterone is essential for meiotic progression of germ cells,

formation and maintenance of the blood-testis barrier, Sertoli cell-spermatid adhesion, and spermiation [10]. Notably, Ar expression in Sertoli cells changes cyclically in correspondence with the stages of seminiferous epithelium development, with peak expression occurring during spermiation [10]. Ar signaling in Leydig cells is crucial for their maturation and regulation of steroidogenic enzymes in adulthood [29]. Furthermore, testosterone signaling in peritubular myoid cells is necessary for maintaining fertility [30].

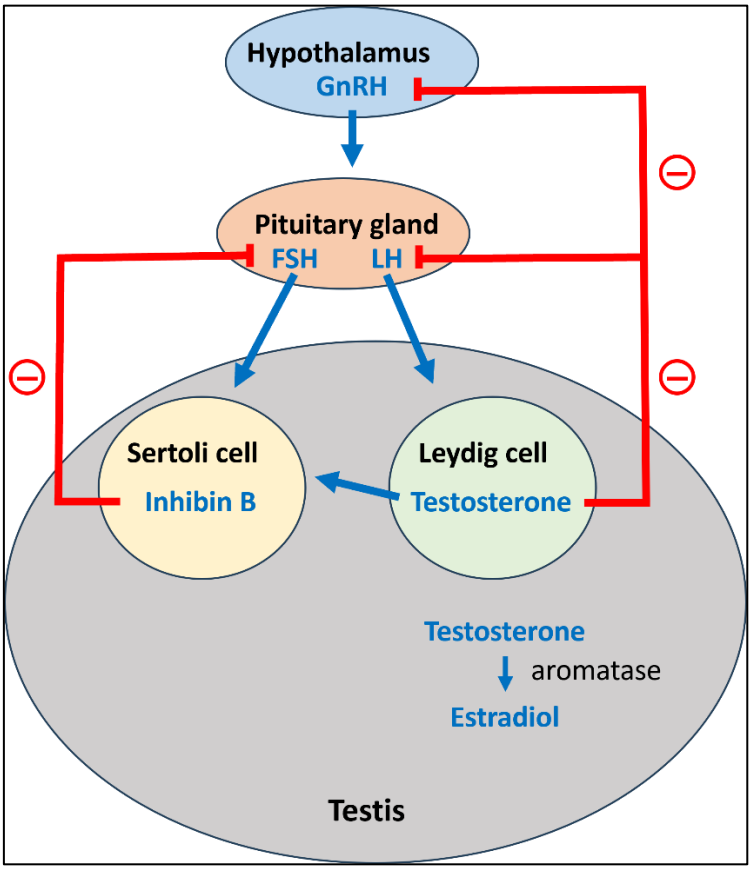


Fig.3. A scheme illustrating the regulatory mechanisms of the hypothalamic-pituitary-gonadal (HPG) axis.

Androgens are vital for male fertility; without testosterone or functional Ar, males are infertile [10]. Androgen signaling during embryonic development is necessary for masculinization. Later in puberty, androgens regulate the development of male secondary sex characteristics, maturation of testicular somatic cells, and activation of spermatogenesis [10, 31].

Part of the testosterone is further metabolized in the testes by aromatase into estrogens, particularly 17β-estradiol (E2). Consequently, estrogen concentrations in the testes are

relatively high, exceeding serum levels found in females [23, 32]. It is therefore not surprising that also estrogens play a crucial role in male reproduction. In recent decades, significant progress has been made in understanding estrogen's role in male reproduction; it has been found to affect various processes, such as proliferation (in gonocytes, spermatogonia, immature Sertoli cells, and Leydig cells), apoptosis (in spermatocytes, spermatids, and Sertoli cells), differentiation (in adult Sertoli cells and round spermatids), spermiation, epididymal maturation, antioxidant defence system, sperm capacitation, and acrosome reaction [23]. The cellular response to estrogen depends on its concentration, the presence of specific types of estrogen receptors (ERs), and the overall cellular context.

Currently, two types of classical ERs are known: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). They can form both homodimers (αα or ββ) as well as heterodimers (αβ). In addition to classical ERs, a membrane-bound estrogen receptor called GPER has been identified in the male reproductive tract [33]. GPER can be located in the plasma membrane [34] as well as in the Golgi apparatus [35] and the endoplasmic reticulum [36]. Most studies agree that ERα is expressed by Leydig cells, ERβ by Sertoli cells, and GPER by both Leydig and Sertoli cells; however, expression patterns for ERα in Sertoli cells and ERβ in Leydig cells are less clear due to conflicting findings across studies [23]. Similarly, there is no consensus regarding estrogen receptor expression in germ cells; detection of ERα, ERβ, and GPER varies depending on the study, species examined, and developmental stage of germ cells [23].

Both testosterone and estrogens are steroid hormones capable of passing through cell membranes to bind with their intracellular receptors. This binding triggers either classical genomic or rapid non-genomic signaling pathway. In classical pathways, androgen or estrogen binding induces conformational changes in their respective receptors (Ar or ER), leading to receptor dimerization followed by translocation to the nucleus where they bind to DNA at androgen response elements (ARE) or estrogen response elements (ERE), thereby regulating gene expression [10, 23]. It has been shown that classical androgen pathway is required for spermatogenesis [10] while classical estrogen pathways are necessary for spermiation [37]. In contrast to the classical pathway, the non-classical pathway results in much faster cellular responses (within seconds to minutes), such as changes in protein phosphorylation or calcium influx; these responses are mediated by membrane or membrane-associated receptors [23]. The rapid non-classical androgen pathway plays a role in remodeling the BTB and sperm

release from Sertoli cells [10]. Regarding estrogen signaling, non-classical pathway is involved in controlling sperm capacitation and the acrosome reaction [38].

The hormonal regulation of male reproduction operates under strict control at multiple levels. The effects of hormones depend not only on their availability but also on the presence of specific receptors and the overall cellular environment. Consequently, a single type of signaling molecule can elicit a variety of cellular responses. Disruption at any level within this regulatory system can lead to diminished sperm quality and quantity, ultimately impairing fertility.

1.3 Endocrine disruptors

Endocrine disruptors (EDs), also known as endocrine-disrupting chemicals, are natural or human-made chemicals that can mimic, block, or interfere with hormones in the body, which are crucial components of the endocrine system. The disruption caused by these chemicals can lead to various adverse health effects, including reproductive impairment [39].

All hormones exert their effects by binding to specific receptors, which initiates distinct intracellular responses that depend on the unique characteristics of the target cell. Specific receptors are key targets of EDs. These chemicals can alter the expression of specific receptors or bind to them, acting as either agonists or antagonists of natural hormones. Additionally, EDs can affect the availability of natural hormones by influencing their synthesis, metabolism, clearance, and distribution, as well as the transport of hormones across cell membranes. EDs may not only affect hormones and their receptors themselves but may also alter signal transduction in hormone-responsive cells or cause epigenetic changes. Finally, these disruptors may impact the fate of hormone-producing or hormone-responsive cells[39].

Different EDs are present in various everyday products, from which they can leach and contaminate water, air, and food. The effects of exposure to EDs can vary based on several factors, including the dose, duration, timing, and route of exposure—whether through ingestion, inhalation, or dermal contact.

Natural hormones function at extremely low serum concentrations, typically in the picomolar to nanomolar range. Similarly, EDs can exert effects at picomolar to micromolar levels [40]. In contrast, toxicological studies often utilize doses in the millimolar range. However, low doses can sometimes have a greater impact on certain endpoints compared to high (toxicological) doses. Low-dose effects were defined by the National Toxicology Program

as those that occur in the range of human exposures or effects observed at doses below those used for traditional toxicological studies [40]. Moreover, exposure to EDs can lead to non-monotonic dose-response effects, where the relationship between dose and effect does not follow the traditional expectation that higher doses produce greater effects. In non-monotonic dose-response curves, the effects observed at low doses can differ significantly from those seen at high doses, making predictions based on high-dose data unreliable [40]. Therefore, to accurately understand the effects of individual EDs on health, it is essential to study their impacts within the range of human and animal exposures.

During male ontogeny, two critical periods of testicular development are particularly sensitive to EDs. The first period occurs during fetal development when the gonads are formed and testosterone production begins. This initial surge of testosterone is essential for the masculinization of the male reproductive system, influencing the development of internal and external genitalia [41]. Following birth, testosterone production ceases and resumes with the onset of puberty, which represents the second critical testicular developmental period. Both developmental stages are crucial for establishing male reproductive capabilities; disruptions during these periods can lead to long-term effects on reproductive health and development.

Epidemiological data consistently indicate a significant decline in both sperm count and quality among men over the past few decades. At the same time, emerging evidence suggests that certain environmental pollutants can adversely affect the male reproductive system and even alter the sperm epigenome. This raises the possibility of a link between rising levels of global environmental pollution and the deterioration of semen quality.

1.3.1 Tetrabromobisphenol A (TBBPA)

Tetrabromobisphenol A (TBBPA) is among the most utilized brominated flame retardants found in the production of epoxy and polycarbonate resin circuit boards, polycarbonate ether, and polyester production. These materials are integral to a wide range of consumer products, including electronics, furniture, plastics, textiles, and kitchen appliances [42]. TBBPA has been introduced to the market as an alternative to bisphenol A (BPA) (Fig.4.), which is a known endocrine disruptor associated with testicular toxicity [43]. The primary routes of exposure to TBBPA are ingestion and inhalation. However, depending on the duration of contact and the dosage involved, TBBPA can also be absorbed through dermal contact [44].

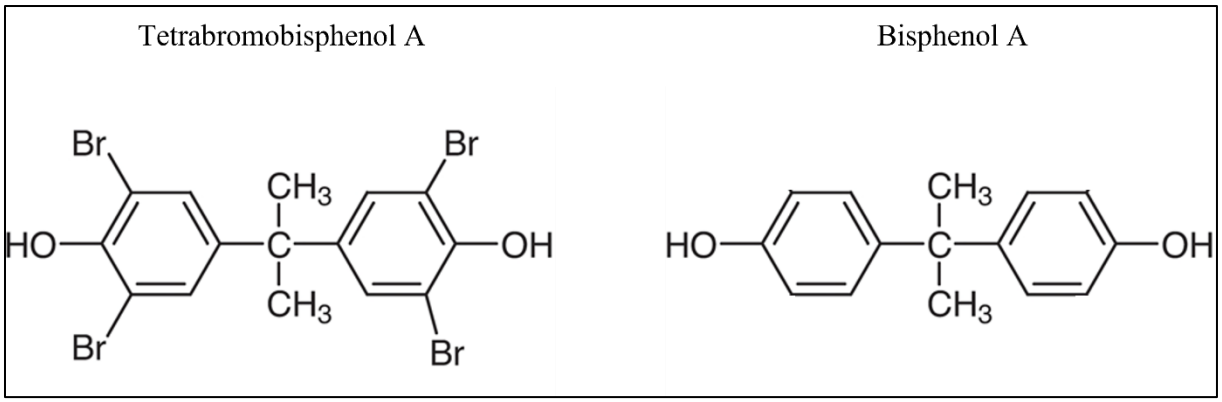


Fig.4. Structures of tetrabromobisphenol A and bisphenol A.

Exposure to TBBPA varies significantly based on geographic location, with higher estimated daily intakes reported in regions such as China, South Korea, and Japan [44]. Research indicates that TBBPA intake is closely related to age; infants and children exhibit the highest exposure levels, with estimates ranging from 0.01 to 3.4 ng/kg b.w./day. In contrast, exposure levels decrease in adolescents and adults, with estimates ranging from 0.003 to 0.61 ng/kg b.w./day [45]. TBBPA has been detected in human plasma samples, breast milk [44], and umbilical cord blood [46]. Furthermore, TBBPA is capable of crossing the placenta, allowing it to reach the fetus [46].

TBBPA has been demonstrated to bind strongly to the thyroid hormone transport protein - transthyretin (TTR) - *in vitro* with an even higher affinity than thyroxine [47]. TTR's primary role is to facilitate the transport of thyroxine and vitamin A within the body. Additionally, TBBPA has been shown to inhibit the binding of triiodothyronine to the thyroid hormone receptor and stimulate the proliferation of GH3 cells, a cell line derived from pituitary tissue that is dependent on thyroid hormones [48, 49]. Beyond its effects on the thyroid hormone system, TBBPA also influences the estrogen hormone system. Studies have revealed that TBBPA and its metabolites can bind to estrogen receptors *in vitro* [50] and induce proliferation of estrogen-dependent cell lines such as MCF-7 [51, 52] and Mit/E2 cells [48].

A study conducted on Wistar rats confirmed that TBBPA can interact with the thyroid hormone system *in vivo*. Moreover, these rats exhibited increased gonad weight and testosterone levels in males, suggesting that the androgen hormonal system may also be affected [53].

Following the publication of our article, additional studies have emerged that contribute to ongoing research in this field. In *in vitro* studies, it has been shown that TBBPA can target spermatogonia, Sertoli cells, and Leydig cells [43] and can act as a human male reproductive toxicant [54]. Specifically, TBBPA affects spermatogonia and primary spermatocytes through perturbation of mitochondrial membrane potential and generation of reactive oxygen species, ultimately leading to apoptosis [54].

Analysis of conventional reproductive endpoints (testicular histology, sperm, and anogenital distance (AGD) — a key indicator of reproductive health) has demonstrated that exposure to TBBPA from *in utero* to adulthood has minimal impact on the male reproductive system in rats [55]. In line with this, it appears that postnatal exposure to TBBPA does not cause adverse effects on conventional reproductive endpoints such as testis weight, AGD, sperm count and motility, or serum testosterone levels in adult male mice. However, this exposure can affect testis development in mice and lead to testicular alterations at the molecular, cellular, and histological levels in adulthood [56, 57]. Specifically, TBBPA exposure resulted in reduced cell proliferation, a decreased number of Sertoli and germ cells, disordered Sertoli cell nuclei arrangement, and cytoskeleton damage by postnatal day 7. This damage to testis development during early postnatal life could contribute to a decreased number of Sertoli cells and spermatogonia, cytoskeleton damage in Sertoli cells, along with downregulated expression of marker genes for Sertoli cells, spermatogonia, and spermatocytes observed in adult testes [57]. Moreover, postnatal TBBPA exposure increases the susceptibility of the testes to future chemical-induced stress, resulting in severe adverse reproductive outcomes [56].

Research on BPA has led to significant regulatory changes, including the prohibition of BPA in baby bottles, cups, and the linings of infant formula cans across many industrialized countries. In response to these restrictions, industries have sought alternative compounds that mimic BPA's properties [55]. One such compound is TBBPA. Given the reproductive health risks associated with BPA, further research is essential to investigate the impact of TBBPA on male reproduction and determine whether it poses potential risks to male reproductive health. The studies referenced above suggest that research on the influence of TBBPA should extend beyond conventional analyses. It is crucial to focus more on its effects at the molecular, cellular, and histological levels.

1.3.2 Zearalenone (ZEA)

Zearalenone (ZEA) is a mycotoxin produced by fungi of the genus Fusarium. It is a common contaminant of cereal grains, and due to its thermostable properties, it can also be found in baked goods. The levels of exposure to ZEA depend on dietary intake and the extent of contamination. Consequently, adults may experience dietary exposure levels to ZEA ranging from 2.4 to 29 ng/kg b.w./day. In contrast, toddlers aged 12 to 36 months typically have average daily intakes between 9.3 and 100 ng/kg b.w./day [58]. The No Observed Adverse Effect Level (NOAEL) for ZEA varies depending on the species studied. In pigs, it has been reported as 40 µg/kg b.w./day, while in rats, the NOAEL is higher at 100 µg/kg b.w./day [59]. Based on the NOAEL for pigs, the European Food Safety Authority (EFSA) has established a tolerable daily intake (TDI) for ZEA of 0.25 µg/kg bw per day.

After oral administration, ZEA is rapidly absorbed from the gastrointestinal tract and metabolized in the liver and small intestine, producing metabolites such as α-zearalenol and β-zearalenol. Both metabolites have a structure similar to estrogen, enabling them to bind to estrogen receptors. This binding results in significant estrogenic activity, which can disrupt hormonal balance and lead to adverse health effects. Consequently, ZEA is classified as an endocrine disruptor [59].

The increase in estradiol levels exerts an inhibitory effect on the pituitary gland, leading to decreased levels of luteinizing hormone (LH). This reduction in LH subsequently results in decreased testosterone production. Since altered testosterone levels can impair spermatogenesis, this mechanism may contribute to the reproductive effects associated with ZEA. Additionally, ZEA can bind to estrogen receptors located on germ cells, Leydig cells, and Sertoli cells, further influencing reproductive function [60].

Research has demonstrated that exposure to ZEA has adverse effects on the reproductive system of adult male mice. Specifically, ZEA treatment leads to a decrease in sperm concentration and quality, which likely contributes to the low pregnancy rates observed in females mated with ZEA-exposed males [61]. Additionally, *in utero* exposure to ZEA results in an increase in AGD, a decrease in sperm quality and count, reduced testosterone levels, and damage to spermatogonia in adult mice [62].

Moreover, *in utero* exposure to ZEA hinders the development of fetal Leydig cells, potentially causing abnormalities in the male reproductive tract [63]. Research conducted by Abidnia et al. [64] attributes the adverse effects of ZEA on testicular structure and function to

alterations in the expression of estrogen receptors (ERα and ERβ). Their findings also suggest that ZEA influences spermatogenesis through mechanisms that partially differ from those of estradiol.

Another study links the reproductive toxicity induced by ZEA to its impact on Sertoli cells. Specifically, ZEA may impair the secretory functions of Sertoli cells by disrupting their cytoskeletal structure and ultrastructure [65]. The effect of ZEA on Sertoli cells has also been investigated in an immature Sertoli cell line (TM4), indicating that ZEA might interfere with Sertoli cell development [66].

Furthermore, ZEA can directly affect sperm. An *in vitro* study has shown that treatment with ZEA or its derivative, α-zearalenol, negatively impacts sperm viability, motility, and the acrosome reaction [67].

While many studies indicate the negative impact of ZEA on male reproduction, it is important to note that these studies were conducted using much higher doses of ZEA than those typically encountered in real environmental exposure. As ZEA is classified as an endocrine disruptor capable of eliciting effects at low doses—effects that cannot be predicted when establishing toxicity limits—it is crucial to test ZEA at concentrations that reflect actual environmental exposure to accurately assess its true impact on health.

1.3.3 Di (2-ethylhexyl) phthalate (DEHP)

Di(2-ethylhexyl) phthalate (DEHP) is one of the most widely used phthalates and is classified as an endocrine disruptor due to its ability to affect hormonal balance, leading to developmental alterations. It is used as a plasticizer in various products, enhancing the flexibility, transparency, and durability of plastics. DEHP is found in everyday items, including food packaging, medical devices, children's products, cosmetics, and various industrial products. The global consumption of DEHP was estimated at 3.07 million tons [68].

Since DEHP is not covalently bound to polyvinyl chloride polymers, it can easily leach out of plastics, contaminating food, blood, air, water, and soil, thereby influencing human and animal health [69]. Given the widespread presence of phthalates in the environment, it is nearly impossible to avoid exposure to them; these substances can enter the body through ingestion, inhalation, and dermal contact [68].

In the body, DEHP is rapidly metabolized to its monoester (MEHP), which can be further biotransformed into oxidative metabolites, glucuronidated, and ultimately excreted in urine [70, 71]. MEHP is ten times more potent than DEHP and accounts for much of DEHP's toxicity [72]. However, not all DEHP and its metabolites are eliminated from the body; DEHP has been found in various biological samples, including rat placenta, fetal tissue, and amniotic fluid [73]. In humans, it has been identified in umbilical cord blood [74, 75], placenta [74, 76], breast milk , and amniotic fluid [70]. Moreover, DEHP and its metabolites can cross the placenta and have direct effects on the developing embryo. Notably, DEHP is more commonly detected in fetal serum compared to maternal serum [74].

The calculated median daily intake of DEHP for humans is approximately 1 – 4.2 µg/kg b.w. [77], which is below the NOAEL for DEHP, determined to be 4.8 mg/kg b.w./day based on monitoring reproductive tract malformations in animals [78]. The NOAEL is used to calculate the Tolerable Daily Intake (TDI), which for DEHP is set at 48 µg/kg b.w./day [79].

Research involving reproductive studies in mice indicates that DEHP is the most potent reproductive toxicant among phthalates [77]. The most frequently observed phenotypes following DEHP exposure include reduced anogenital distance, decreased weight of reproductive organs, undescended testes, lower daily sperm production, testicular and epididymal malformations, and altered steroid hormone levels [80-86]. DEHP has been demonstrated to impact not only sperm concentration but also sperm quality [87-89]. It disrupts the differentiation of Sertoli cells [90] and modifies the expression of various testicular genes [91, 92]. Moreover, high doses of DEHP during gestation have been shown to cause embryotoxicity and teratogenic effects in both mice and rats [93, 94].

Furthermore, DEHP can affect both the development of sperm and the sperm cells themselves. Incubation of sperm with 10 µM DEHP significantly disrupts maturation processes, specifically altering sperm capacitation-associated phosphorylation kinetics, impairing the acrosome reaction, and increasing reactive oxygen species production [88]. *In vitro* incubation of human and dog sperm with DEHP has been shown to increase DNA fragmentation and decrease sperm motility [95]. Another study demonstrated a link between ambient air concentrations of DEHP and adverse effects on sperm motility and chromatin DNA integrity in men [89].

Research indicates that the adverse effects of DEHP can also be transgenerational [96], with paternal versus maternal transmission playing a significant role [97]. Specifically, prenatal

DEHP exposure has been associated with decreases in serum testosterone, fertility, sperm concentration, motility, and histopathological changes in the seminiferous tubules of paternal F3 lineage males [97]. Notably, the adverse effects of DEHP exposure were more severe in males from the paternal F3 line compared to those from the maternal F3 line. Interestingly, a lower concentration of DEHP (20 μg/kg/day) resulted in more severe effects than a higher concentration (200 μg/kg/day) [97].

An intriguing discovery emerged from a long-term study examining various concentrations of DEHP, revealing that prenatal exposure to DEHP leads to premature reproductive aging in male mice [85]. While testosterone concentrations in DEHP-treated mice were like those of control mice when examined at postnatal day 21 (PND 21), all DEHP-exposed males exhibited reduced serum testosterone levels by 16 months of age [85]. This underscores the importance of the age at which analysis is conducted.

When investigating the effects of DEHP in animal models, it is essential to recognize that not all mouse strains exhibit the same level of sensitivity. For instance, FVB/N mice show lower sensitivity to DEHP compared to C57BL/6J mice [82].

In humans, DEHP exposure has been linked to increased sperm DNA fragmentation and decreased sperm motility [89]. Furthermore, prenatal levels of some metabolites of DEHP appear to be negatively associated with reproductive function in adolescent males [98].

The effects of DEHP are influenced by several factors, including dose, timing and duration of exposure, the age of the individuals studied, and individual sensitivity to the compound. It is widely believed that DEHP functions as an anti-androgen, thereby influencing androgen-mediated biological processes. However, it is important to note that most research has utilized doses significantly higher than those typically encountered in daily human exposure. This discrepancy raises concerns, particularly because some chemicals may exhibit a nonlinear dose-response relationship. In such cases, lower doses can have a more pronounced effect on certain biological endpoints compared to higher, potentially toxic doses [40]. To accurately evaluate the true health risks associated with DEHP, it is essential to conduct studies using concentrations that reflect actual human and animal exposure levels. This approach will provide a more comprehensive understanding of DEHP's impact on health.

2 Aims of the work

- To evaluate the effect of increased 17β-estradiol concentration on the capacitation status of mouse sperm

- To compare the methods used for detecting sperm capacitation and evaluate their ability to predict the acrosome reaction induced by the *zona pellucida*

- To assess the impact of selected endocrine disruptors on male reproductive health, including sperm and testicular physiology as well as testicular gene expression, at doses that reflect typical human exposure levels

3 Results and discussion

3.1 Sperm capacitation: the effect of natural estrogen on sperm capacitation and fluorescent analysis of this process (Supplements 1 and 2)

3.1.1 The *in vivo* effect of 17β-estradiol on sperm capacitation in the cauda epididymis

Natural estrogens play a crucial role in the regulation of the female estrous cycle. However, these important "female" hormones also regulate sperm production and maturation. Estrogens are synthesized at relatively high levels in the testes, where they regulate spermatogenesis and subsequently facilitate sperm passage through the epididymis. Furthermore, sperm encounters estrogens in the female genital tract, where it undergoes a series of maturation changes collectively called capacitation.

Correct hormonal balance is crucial for successful reproduction, as changes in hormone levels can disrupt spermatogenesis and the maturation processes of sperm, including epidydimal maturation and capacitation. Such disruptions can ultimately reduce reproductive potential. Nowadays, humans are exposed to numerous estrogenic compounds that may disturb this hormonal balance. Research has shown that estrogens have a pro-capacitation effect that is concentration-dependent [99]. However, much of the knowledge regarding the impact of estrogens on sperm capacitation comes from *in vitro* studies that simulate estrogen action on sperm within the female reproductive tract.

In our study, we aimed to determine whether elevated estrogen levels in males could also affect sperm capacitation. We exposed male mice to 17β-estradiol (E2) at a concentration of 20 ng/ml per day, either during puberty or continuously. Upon reaching adulthood, the male mice were euthanized, and their testicular and epididymal sperm were analysed for capacitation status through the detection of tyrosine phosphorylation and the CTC assay (Fig.5).

We did not observe any alterations in the capacitation status of testicular sperm from E2-treated males compared to the control group. However, the percentage of capacitated sperm released from the epididymis was significantly increased in E2-treated mice. This effect of E2 appears to be reversible, as the percentage of capacitated sperm decreased after the termination of E2 exposure (pubertal vs continuous group). Additionally, E2 serum levels were elevated in the continuous treatment group, while they remained relatively similar in the control and pubertal groups. We also measured the expression of the TFF1 gene, a specific estrogen-

responsive gene in testicular tissue. Both E2 concentration and TFF1 gene expression showed a positive correlation with the percentage of capacitated sperm.

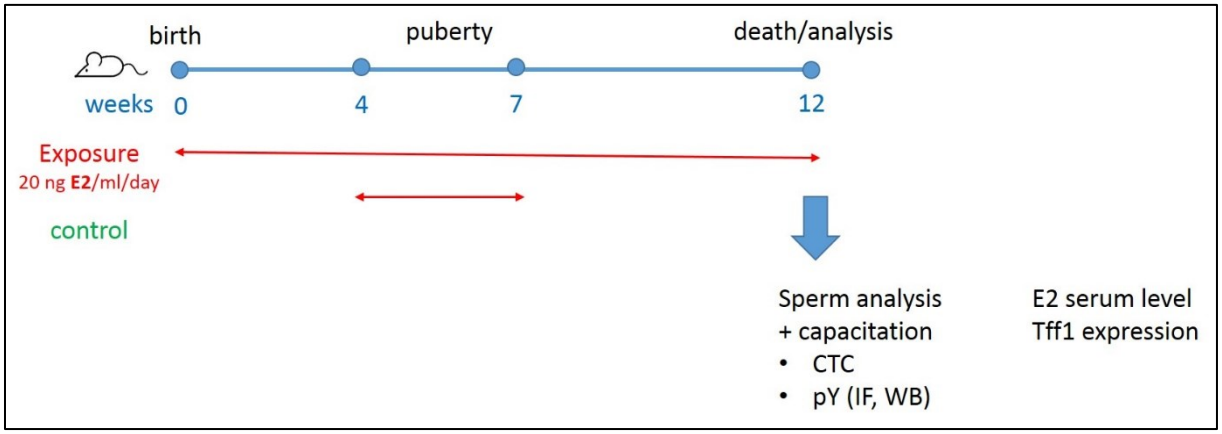


Fig.5. Diagram of experimental design.

To determine whether the pro-capacitation effect of estrogen will be propagated further, we capacitated epididymal sperm *in vitro*. However, the differences between the control and experimental groups remained consistent with those observed at the beginning.

Changes in sperm maturation can affect the sperm's ability to be in the right place at the right time to fertilize the egg. Therefore, the observed premature sperm capacitation in the cauda epididymis following *in vivo* exposure to E2 may have harmful effects on reproductive fitness. Moreover, since certain EDs possess estrogenic activity, this may represent one of the pathways through which these substances contribute to the increasing prevalence of fertility issues.

3.1.2 Fluorescent analysis of the *in vitro* capacitation process in boar spermatozoa

The study of capacitation, which is a key prerequisite for successful egg fertilization, strongly depends on the appropriate choice of detection method. In our second study, we tested several fluorescent techniques to evaluate their ability to detect changes in various molecular processes during the capacitation progress. Additionally, we aimed to determine their effectiveness in predicting the percentage of acrosome-reacted sperm following exposure to solubilized *zona pellucida*.

We incubated boar sperm in a capacitating medium and assessed the capacitation status at several incubation times, including after the induction of the acrosome reaction. To evaluate

the capacitation status of the sperm, we employed four widely used methods for studying sperm capacitation, each targeting different molecular characteristics: **1. Chlortetracycline (CTC) assay** detects changes in the distribution of intracellular calcium in the sperm head during capacitation; **2. Anti-acrosin antibody (ACR.2) assay** detects rearrangements in the plasma membrane and acrosomal matrix. The increased accessibility of acrosin epitopes serves as a marker of capacitation progress; **3. Anti-phosphotyrosine (pY) antibody assay** detects increase in protein phosphorylation that occur during capacitation; **4. Fluorescein isothiocyanate-conjugated phalloidin (FITC-phall) assay** recognizes the rearrangement of the cytoskeleton by detecting F-actin. The four tested methods were evaluated using fluorescent microscopy and flow cytometry (Fig.6).

We confirmed that all the methods compared are suitable for evaluating capacitation using fluorescent microscopy. However, a significant disadvantage of fluorescent microscopy is its inherent subjectivity. For instance, the pY and Phall assays lack a specific fluorescent pattern that corresponds to the progress of capacitation. These methods rely on detecting changes in fluorescent intensity during capacitation, which necessitates setting an intensity threshold.

The CTC assay is the most widely used method for studying capacitation. Its main advantage is the presence of a specific fluorescent pattern corresponding to distinct stages of the capacitation process. However, its relatively low fluorescent intensity and fast photobleaching can make analysis challenging for the human eye. In contrast, the ACR.2 antibody analysis shows great promise, as capacitated sperm can be clearly distinguished by a strong positive signal and a prominent acrosomal pattern.

Subjective microscopic analysis can be overcome by using flow cytometry, which allows for rapid analysis of large numbers of cells and can accurately measure small differences in fluorescent intensity. We achieved the best results with the ACR.2 method, which displayed three well-separated peaks corresponding to non-capacitated, capacitated, and acrosome-reacted sperm. Conversely, the CTC method was unsuitable for flow cytometric analysis because its fluorescent intensity was highly stochastic and did not yield three distinguishable peaks. The pY and Phall detection methods produced three distinguishable peaks, but these were not completely separated (Fig.6., right part).

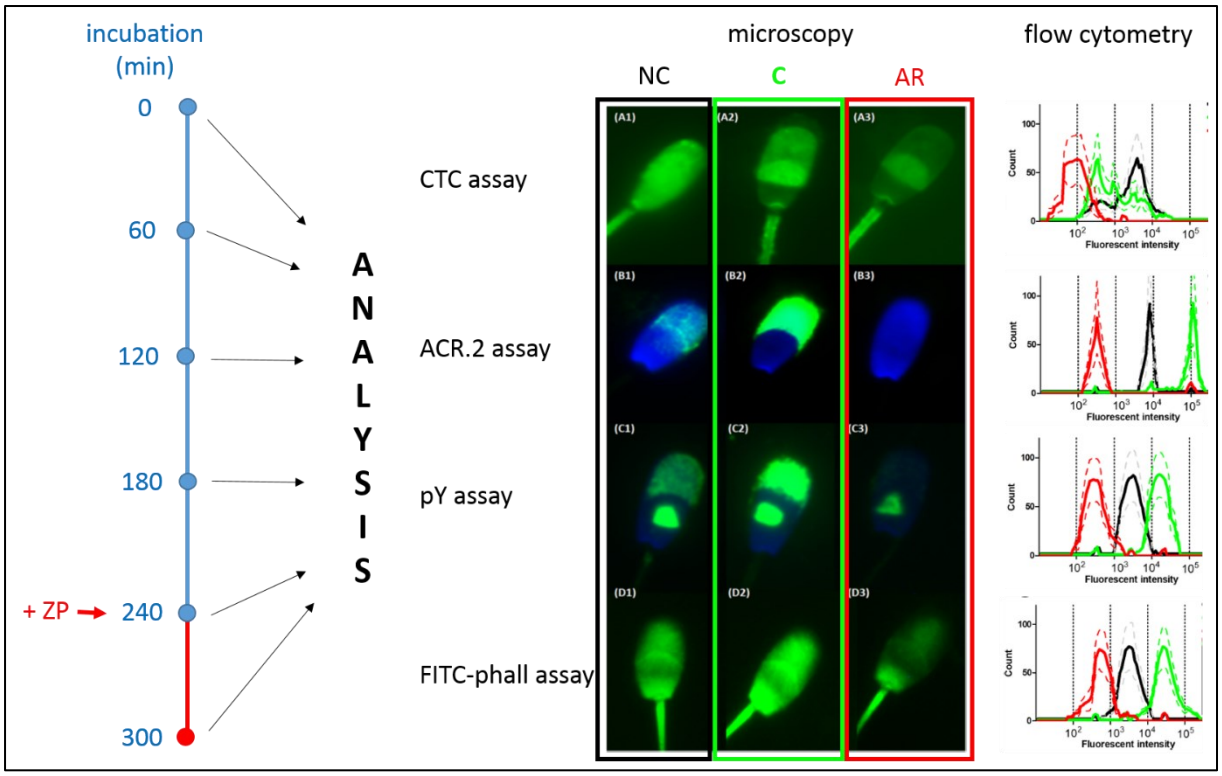


Fig.6. Diagram of experimental design. Left part – boar sperm incubation timeline; right part – representative fluorescent patterns of capacitated sperm observed under the microscopic evaluation, and histograms from the flow cytometry analysis obtained using different assays. NC – negative control, C – capacitated sperm, AR – acrosome-reacted sperm

Overall, the flow cytometry data aligned well with those obtained from fluorescent microscopy (Tab.1.). Although there was significant correlation between the data from individual methods, notable differences existed in their correlation coefficients. The highest correlation in the number of capacitated sperm cells among the detection methods was observed between fluorescent microscopy detection using CTC and the ACR.2 antibody ($r = 0.81$). High correlation was also noted between ACR.2 (FC, FM) and CTC (FM), while a low correlation was found for pY (FM) and Phall (FM), with the lowest correlation for CTC (FC). This indicates that for some methods, flow cytometry is more appropriate than fluorescent microscopy (e.g., pY, Phall), while the opposite is true for CTC. This consideration should be factored into experimental design.

	ACR.2 FM	ACR.2 FC	CTC FM	CTC FC	Phall FM	Phall FC	pY FM	pY FC
ACR.2 FM[a]	**1**	**0.79**	**0.81**	0.19	**0.33**	**0.55**	**0.47**	**0.55**
ACR.2 FC[b]	**0.79**	**1**	**0.69**	0.17	**0.33**	**0.62**	**0.39**	**0.44**
CTC FM[c]	**0.81**	**0.69**	**1**	0.25	**0.35**	**0.59**	**0.52**	**0.47**
CTC FC[d]	0.19	0.17	0.25	**1**	0.24	0.31	0.12	0.23
Phall FM[e]	**0.33**	**0.33**	**0.35**	0.24	**1**	**0.47**	0.27	**0.35**
Phall FC[f]	**0.55**	**0.62**	**0.59**	0.31	**0.47**	**1**	0.23	0.29
anti-pY FM[g]	**0.47**	**0.39**	**0.52**	0.12	0.27	0.23	**1**	**0.71**
anti-pY FC[h]	**0.55**	**0.44**	**0.47**	0.23	**0.35**	0.29	**0.71**	**1**
r total[i]	**4.69**	**4.43**	**4.68**	**2.51**	**3.34**	**4.06**	**3.71**	**4.04**

Tab.1. Correlation of individual detection methods for boar sperm capacitation. Green box – high correlation; red box – low correlation.

Combining data from fluorescent microscopy and flow cytometry allows us to describe the temporal sequence of molecular processes detected by individual analytical method. The first observable molecular changes included calcium redistribution (CTC) and rearrangement of the acrosomal matrix (ACR.2). Increases in tyrosine phosphorylation (pY) and actin polymerization (Phall) were observed at later stages of capacitation. It is important to note that we detected molecular changes at only five different time points during capacitation. Using life imaging would be more appropriate for characterizing the sequence of molecular processes during capacitation in detail. Furthermore, monitoring multiple parameters simultaneously within a single cell would provide additional insights.

During capacitation, sperm acquire the necessary properties to reach and bind to the egg, as well as to undergo the acrosome reaction, which is an absolute prerequisite for successful fertilization. The natural inducers of the acrosome reaction are the *zona pellucida* and progesterone. In our next step, we tested the ability of individual methods to predict the percentage of acrosome-reacted (AR) sperm following exposure to solubilized *zona pellucida*. We correlated the percentage of capacitated sperm detected by individual methods after 240 minutes of capacitation with the percentage of AR sperm, which was assessed by staining the acrosome with Pisum sativum agglutinin.

Our results indicate that all tested methods demonstrate a good correlation with the number of sperm undergoing *zona pellucida*-induced acrosome reaction. Among them, the CTC (FM) and ACR.2 (FM) methods were found to be the most accurate in predicting the percentage of sperm that undergo the acrosome reaction. In contrast, the pY and Phall methods considerably underestimate the percentage of sperm that will undergo the acrosome reaction induced by the *zona pellucida*. This discrepancy should be considered when studying

capacitation and the effects of various substances on both capacitation and the acrosome reaction.

3.2 The impact of low concentrations of selected endocrine disruptors on male reproductive health (Supplements 3, 4, 5)

Male reproduction is regulated by the endocrine system, including estrogen signaling. Maintaining a proper hormonal balance is crucial for normal physiological function. However, numerous substances in our environment can mimic natural hormones and disrupt physiological processes. These substances are known as endocrine disruptors (EDs).

Studying EDs is essential to uncover their harmful effects on human and animal health, which may lead to the introduction of legislative measures. Toxicological studies often utilize doses that are many times higher than typical human exposure to EDs. However, it is recognized that some substances can exert greater effects at lower concentrations compared to higher doses. Furthermore, the impact of EDs can be transmitted to subsequent generations, and continuous exposure across generations may lead to an accumulation of negative effects.

The second part of presented work focuses on evaluating the effects of three known endocrine disruptors on male reproduction at doses relevant to human intake. In the case of TBBPA, we also examine transgenerational transmission. These chemicals were selected as examples of substances that function as anti-androgens (DEHP) or exhibit estrogenic activity (ZEA). The last chemical, TBBPA, represents an alternative that has replaced the well-known endocrine disruptor associated with testicular toxicity—BPA.

3.2.1 The impact of tetrabromobisphenol A on male reproduction: specifically, the induction of apoptosis and testicular gene expression

The first endocrine disruptor studied was tetrabromobisphenol A (TBBPA), a substance commonly used in industry as a flame retardant. Research has demonstrated that TBBPA impacts the thyroid hormone system [47-49, 53], as well as estrogen [48, 50-52] and androgen hormone systems [53]. Additionally, because TBBPA can be transported across the placenta, it may also affect fetal development [46].

In our study, we aimed to investigate the effects of long-term exposure to TBBPA on male reproduction. To achieve this, we conducted a two-generational *in vivo* study using mice, with continuous exposure to TBBPA at a dose of 35 μg/kg b.w./day. In the parental generation, females from the experimental group were exposed to TBBPA during gestation, while control animals were not exposed. The parental generation was bred to produce the F1 generation, which consisted of two groups: the control group (**C**), where mice were not exposed to TBBPA, and the experimental group (**T**), where mice were continuously exposed to TBBPA from foetal development to adulthood. The F1 generation was bred as follows: mothers and fathers from group C formed group **CC**; both parents from group T formed group **TT**; mothers from group C and fathers from group T formed group **CT**; and mothers from group T and fathers from group C formed group **TC**. Pups in the F2 generation were exposed to TBBPA only in groups TT and TC. All animals were euthanized at the same age of 70 days (Fig.7.).

TBBPA had no effect on the number of progeny, sex ratio, anogenital distance (AGD), body weight, or sperm quality in either generation. This observation is consistent with a later studies that also did not find any adverse effects of postnatal exposure to TBBPA on conventional reproductive endpoints in adult mice [56, 57]. Examination of organ weights revealed a reduction in testicular weight, along with an increase in the weight of the seminal vesicles and prostate in the **TT** group. Additionally, there was an increased weight of the epididymis in the **CT** group.

We did not observe any pathological changes in the morphology of the seminiferous tubules or spermatogenesis. However, morphometric analysis revealed a lower thickness of the seminiferous epithelium in the **T**, **TC**, and **TT** groups. Mice from these groups exhibited an increased number of apoptotic cells in the testes, along with altered expression of apoptotic genes (*Bax* and *Bcl2*) and decreased expression of the Sertoli cell-specific gene (*Sox9*). Previous studies have shown that TBBPA induces apoptosis in TM4 Sertoli cells *in vitro* [100]. We hypothesize that the reduced thickness of the seminiferous epithelium is primarily due to TBBPA-induced apoptosis of Sertoli cells. Recent research has demonstrated that TBBPA adversely affects testis development during early postnatal life, particularly impacting Sertoli cells and germ cells. This damage leads to a reduction in the number of Sertoli cells and spermatogonia, as well as cytoskeletal impairment in Sertoli cells. Furthermore, there is a down-regulation of key marker genes associated with Sertoli cells (such as *Sox9*), spermatogonia, and spermatocytes in adulthood [57].

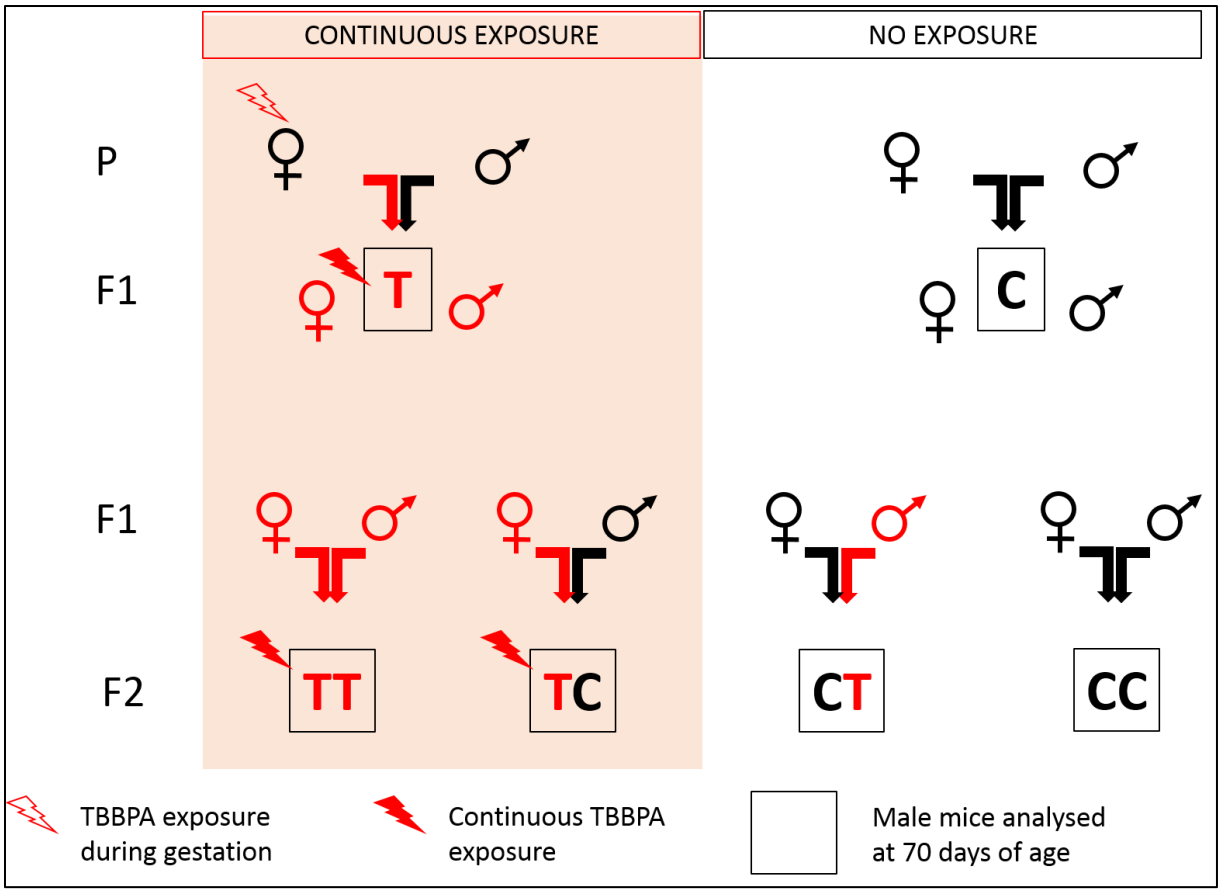


Fig.7. Diagram of experimental design. The red color indicates animals that were exposed to TBBPA during their lifetime, while the black color indicates animals that were not exposed. T – TBBBPA exposed mice, C – control mice.

Gene expression analysis of several genes for heat shock proteins (*Hsp70-2*, *Hsc70t*, *Hsp60*, *APG-1*) confirmed that TBBPA induced stress in testicular cells. Interestingly, decreased expression of *Hsp70-2* and *Hsp60* was also detected in mice not directly exposed to TBBPA (only their fathers were; group **CT**). Furthermore, expression of the androgen receptor (*Ar*) was downregulated only in the second generation within the **TT** and **TC** groups. All the results are summarized in Fig.8.

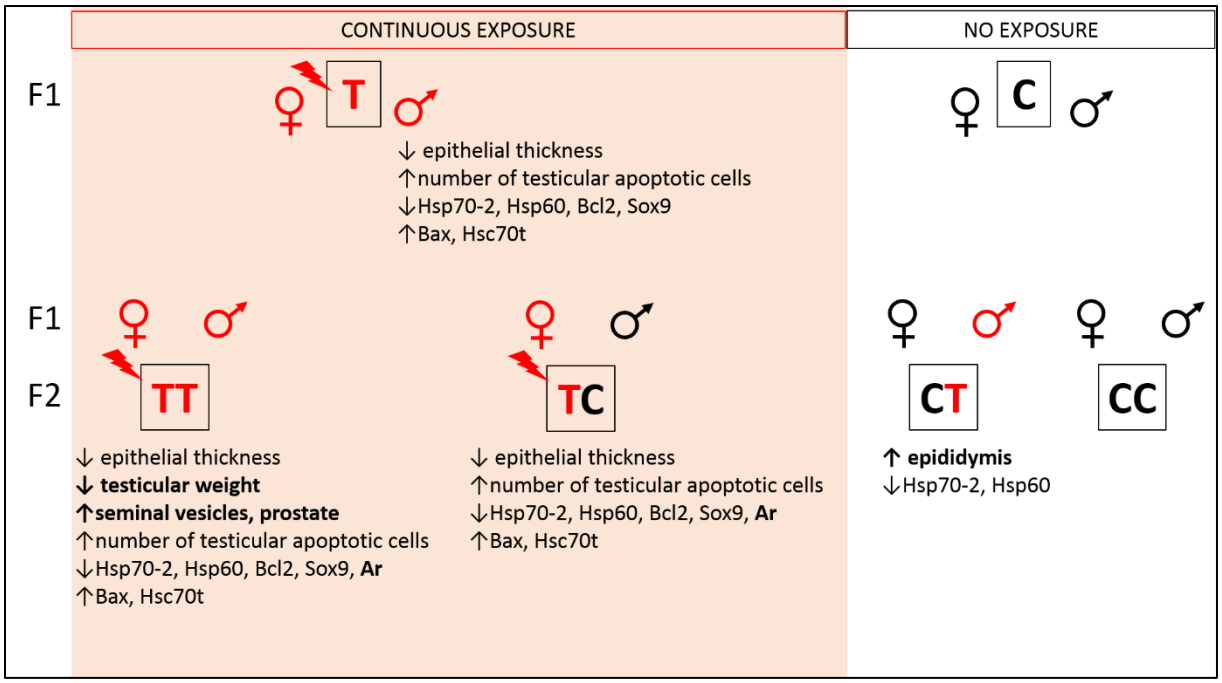


Fig.8. Summary of the results. The red color indicates animals that were exposed to TBBPA during their lifetime, while the black color indicates animals that were not exposed. T – TBBBPA exposed mice, C – control mice.

In general, the groups most affected were those administered TBBPA (T, TC, and TT). Furthermore, TBBPA had a more pronounced effect on the second generation compared to the first. Additionally, within the second generation, the mice from the TT group (whose both parents were exposed to TBBPA) were more affected than those from the TC group (whose mothers were exposed to TBBPA). This finding, along with the observation of effects in the CT group—where male mice were not directly exposed to TBBPA, but only their fathers were—suggests that TBBPA may induce epigenetic changes that are transmitted to subsequent generations.

3.2.2 The impact of zearalenone on reproductive parameters and selected testicular gene expression in mice

The second endocrine disruptor studied was the potent estrogenic, heat-stable mycotoxin Zearalenone (ZEA). ZEA is produced by *Fusarium* fungi and is a common contaminant of cereal crops worldwide. Its primary effect arises from its competition with the natural hormone estrogen for estrogen receptors. Consequently, it is not surprising that ZEA has been detected

in estrogen target tissues such as the uterus, ovarian follicles, and adipose tissues, as well as in the testes [101].

Numerous studies have highlighted the adverse effects of ZEA on male reproductive health. However, it's important to note that these studies typically used doses of ZEA than those expected in real environmental exposure. ZEA is recognized as an endocrine disruptor, which means it can produce effects at low doses that are difficult to predict when tested at the higher doses typically used in traditional toxicological studies. Therefore, in our study, we aimed to assess whether low doses of ZEA also have an effect on male reproduction.

We used concentration relevant to human daily exposure (0,15 µg/l ~ 25 ng/kg b.w.; low dose) and a concentration 1000 times higher (150 µg/L; high dose). Mice were continuously exposed to these concentrations throughout their lives, starting from the first day of the mothers' pregnancy, and were analyzed as adults at 70 days of age. The diagram of the experimental design is depicted in Figure 9.

ZEA exposure had no effect on the sex ratio, number of progeny, AGD, body weight, reproductive organ weight, or the morphology of seminiferous tubules at either concentration. However, we observed decreased sperm quality in both exposed groups, with a more pronounced impact at the low dose, where a reduction in sperm concentration was also detected. The impact of ZEA on sperm quality and concentration was also observed in toxicological studies [61, 62].

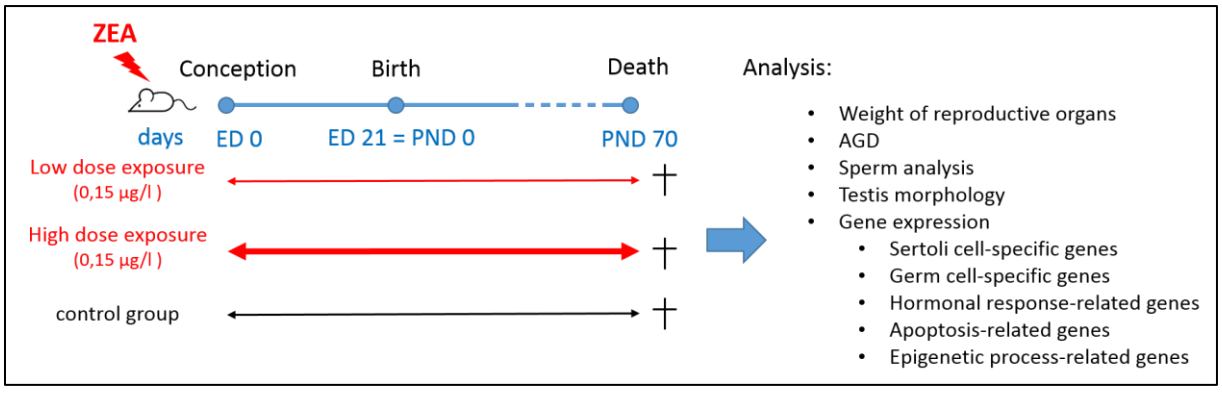


Fig.9. Diagram of experimental design. ED – embryonic day; PND – postnatal day.

Additionally, we analyzed 28 testicular genes involved in testicular physiology and spermatogenesis. All the results are summarized in Figure 10. Similar to the findings regarding sperm quality, the lower dose had a more significant effect on gene expression. We found altered expression of germ cell-specific genes only in the group exposed to the low concentration of ZEA. Specifically, we detected decreased expression of *Vegfa*, a gene expressed specifically in spermatogonia; decreased expression of spermatocyte-specific genes—*Sycp3*, *Ccna1*, and *Grth*; and increased expression of *Sycp1*, another gene expressed in spermatocytes. The expression of tested round spermatid-specific genes (*Prm1, Tnp1, Tnp2*) remained unchanged.

Regarding genes specifically expressed in Sertoli cells, we observed decreased expression of *Sox9* in both experimental groups. In the low-dose group, there was increased expression of *Mas1*; in the high-dose group, we noted decreased expression of *Wt1*; and there were no changes in the expression levels of *Eps8* and *Icap1*. Later studies have linked the toxic effects of ZEA on reproduction to impairments in Sertoli cell function [65]. Additionally, it was found that ZEA may interfere with Sertoli cell development [66].

For genes involved in hormonal response, we detected decreased expression of androgen receptor (*Ar*) and its component *Fkbp5* in the low-dose group. Although ZEA is known for its estrogenic effects, we did not observe changes in genes related to estrogen response (*Tff1*, *Igfbp5*, *Ctsd*). However, we did not analyze the expression of the estrogen receptors themselves. Previous studies have shown that exposure to ZEA leads to alterations in the expression of estrogen receptors (ERα and ERβ) [64]. Finally, exposure to ZEA did not alter the expression of *Fshr*.

We also examined apoptosis-related genes: *p53* and *p21* were downregulated, while *Bcl2* remained unchanged in both experimental groups. This finding is consistent with the absence of any observed alternation in the apoptosis of testicular cells.

Finally, exposure to ZEA may influence epigenetic processes, as indicated by changes in gene expression. We noted downregulation of *Ccnd1*, *Spata2*, *Kdm4a* in animals exposed to the low dose of ZEA and downregulation of *Ccnd1*, *Dnmt1* in those exposed to the high dose.

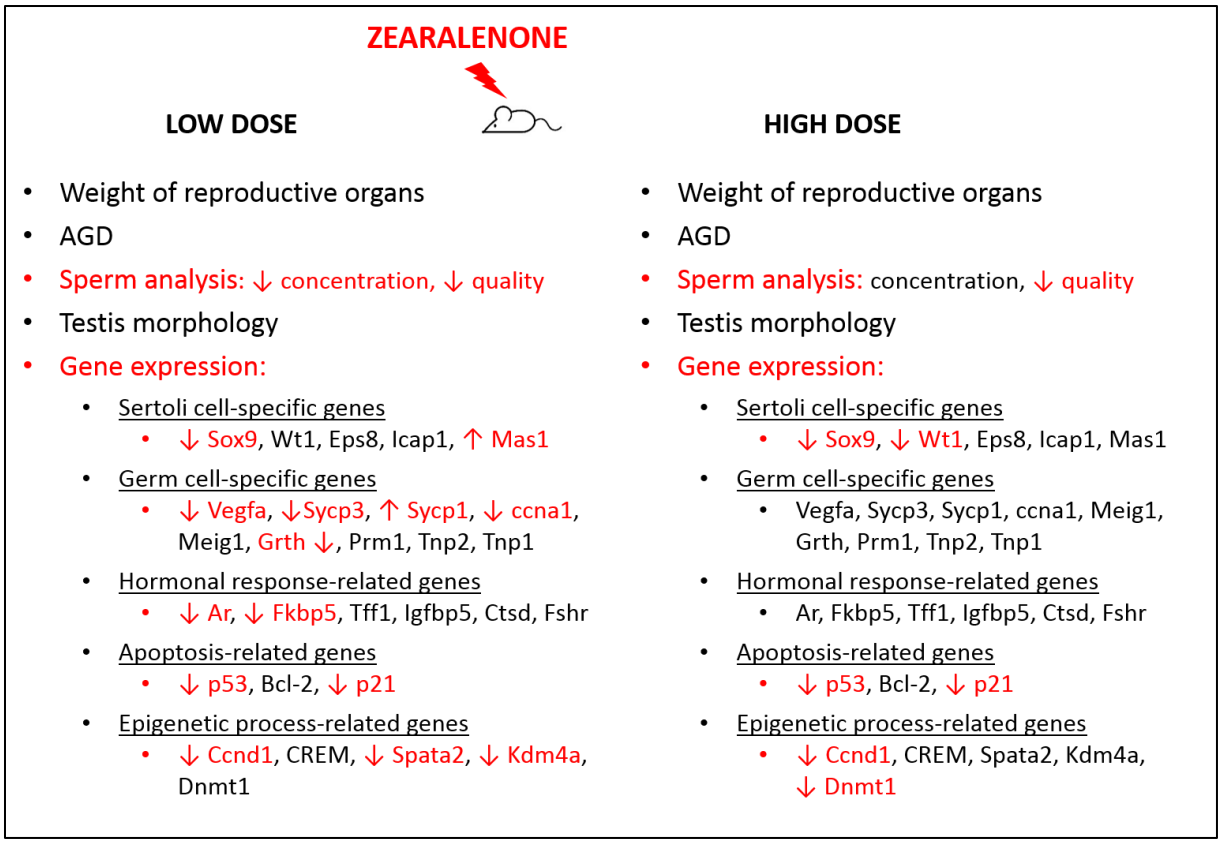


Fig.10. Summary of results from the analysis of male mice exposed to low, environmentally relevant, and high dose of ZEA. Results highlighted in red indicate the changes observed compared to control mice.

Our findings suggest that the reduced sperm count may originate from disruptions at the level of spermatogonia, particularly affecting their self-renewal and differentiation processes, as well as impacting spermatocytes through the modulation of several key meiotic genes. This may be the result of impaired Sertoli cell function. Interestingly, we did not observe any significant effects of ZEA on the expression of genes related to estrogen signaling; instead, it was the androgen-related genes that were affected.

Furthermore, it is worth noting that males exposed to the low concentration of ZEA were considerably more affected than those exposed to the high dose. Given that the lower dose of ZEA utilized in our study reflects realistic human exposure levels, it is crucial to investigate the effects of ZEA at concentrations that accurately reflect these real-world conditions. This approach will help elucidate the true impact of ZEA exposure on male reproductive health.

3.2.3 The impact of di-(2-ethylhexyl) phthalate exposure during gestation and puberty on male reproduction

The third endocrine disruptor studied was di-(2-ethylhexyl) phthalate (DEHP), a compound widely used as a plasticizer. It is known that DEHP can leach from plastics into the environment and influence human health, including embryonic development, as DEHP and its metabolites can cross the placenta [74]. Based on reproductive studies in mice, DEHP is considered the most potent reproductive toxicant among phthalates [77]. However, these studies were conducted at high doses of DEHP and may not fully reflect the actual risk of DEHP exposure to humans and animals. The calculated daily DEHP intake for humans is below the no-observed-adverse-effect level for this compound. Therefore, we aimed to determine whether exposure to a low dose of DEHP, relevant to human exposure, during two critical developmental periods could also lead to reproductive impairment.

We exposed mice to DEHP (2.4-3 μg/kg b.w./day) during the masculinization programming window (gestational days 10-20; group G) or postnatally during puberty (postnatal days 21-45; group P). Male mice without treatment formed the control group (C). All mice were euthanized at the same age of 70 days. The diagram of the experimental design is depicted in Figure 11.

Similar to studies conducted with higher doses of DEHP [80-86], we observed reduced AGD, decreased weight of seminal vesicles (statistically significant only in group P), decreased sperm concentration, and impaired sperm quality, including morphology and acrosome integrity, in both exposed groups. Furthermore, an increased number of apoptotic sperm was detected in group G. On the other hand, we did not observe any impairment in testicular morphology; likewise, the germ cell-specific gene expression analysis did not indicate any disturbance in spermatogenesis. Additionally, testicular cell apoptosis did not change in the exposed mice. In contrast to our findings all these parameters were influenced by high doses of DEHP. It remains an open question whether we would find altered testicular morphology if we analyzed the mice at a later age, similar to the study on prenatal DEHP exposure [85].

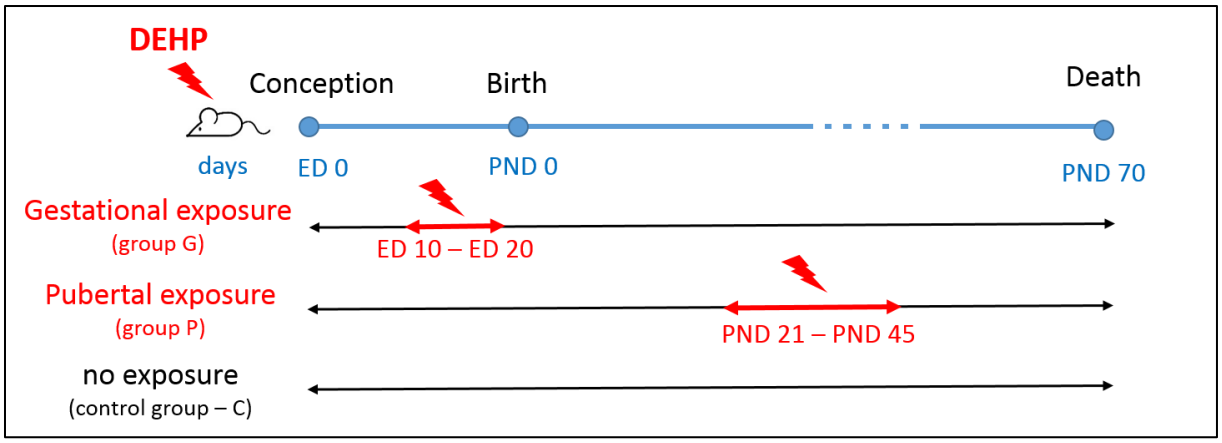


Fig.11. Diagram of experimental design. ED – embryonic day; PND – postnatal day.

AGD and the weight of seminal vesicles are androgen dependent. The observed alterations in these parameters suggest a disruption of androgen signaling during the masculinization programming window and puberty. The conducted gene expression analysis revealed that steroidogenesis and androgen signaling are affected. Similar to the results observed for AGD and the weight of seminal vesicles, DEHP had a greater effect on animals exposed during puberty, with all tested genes being downregulated.

The regulation of endocrine function was affected solely by pubertal exposure to DEHP. The observed decreased expression of the LH receptor (*Lhcgr*) may be a cause or contribute to lower steroidogenesis. Additionally, estrogen, thyroid hormone, and FSH signaling may also be affected by DEHP.

Sertoli cells play a critical role in maintaining spermatogenesis, with their proliferation and maturation being regulated by androgen signaling [102]. Additionally, androgen signaling also governs the permeability of the blood-testis barrier (BTB) [103]. We did not observe any changes in the number of Sertoli cells in the exposed mice. However, we detected a decreased expression of *Gja1* (involved in the formation and regulation of the BTB) and *Eps8* (which plays a role in apical ectoplasmic specialization) in mice exposed during puberty, indicating a disruption of Sertoli cell function.

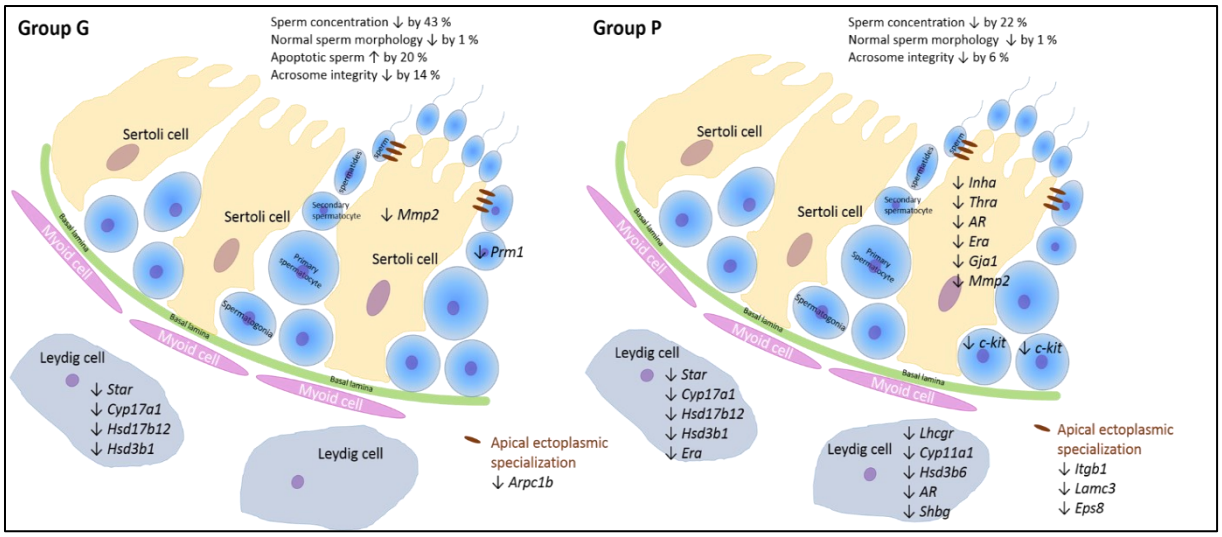


Fig.12. Summary of results obtained from analysis of prenatally (group G) and postnatally (group P) DEHP exposed male mice. These pictures are showing drawing of testicular tissue and different cell population present in testes. Data from analysis of gene expression and sperm parameters in animals are shown in respective cell types.

To further investigate, we conducted an analysis of genes whose protein products are involved in the formation and regulation of blood-testis barrier (*Cldn11, Ocln, Tjp1*), as well as those related to ectoplasmic specializations and tubulobulbar complexes (*Ncad, Itgb1, Lamc3, Mmp2, Timp2, Tes, Arpc1b, Eea1*). Although it is known that androgen signaling in Sertoli cells regulates the permeability of the BTB, and previous studies have shown that DEHP exposure causes slight functional damage to the BTB [104], we did not observe any changes in the expression of genes whose protein products form the BTB. On the other hand, we found decreased expression of genes related to the formation of apical ESs (*Itgb1, Lamc3*) in group P and downregulation of *Arpc1b* (associated with TBC formation and BTB integrity) in group G. Additionally, *Mmp2* was downregulated in both experimental groups. MMP2 regulates apical ES degeneration and subsequently BTB remodeling. These results indicate that Sertoli cell function is impaired. Since the expression of most of these tested genes is regulated by testosterone, it remains to be determined whether the observed effects are related to decreased androgen signaling or whether DEHP has a direct impact on Sertoli cell function.

Overall, we observed a similar trend across all analyzed parameters in both DEHP-exposed groups. However, *in utero* exposure resulted in more extensive damage to sperm quality and concentration compared to pubertal exposure. In contrast, we found more significant changes in gene expression levels in animals exposed during puberty (Fig.12.).

We hypothesize that exposure to environmentally relevant dose of DEHP during sensitive developmental periods, such as fetal development and puberty, impairs the maturation of Leydig and Sertoli cells. This impairment may trigger epigenetic changes that damage Leydig and Sertoli cell function in adulthood. Altered androgen signaling, likely resulting in damage to ectoplasmic specialization and the BTB, contributes to the observed decreases in sperm concentration and quality. Furthermore, the increased number of apoptotic sperm may suggest potential impairment in epididymal maturation or sperm storage.

4 Conclusion

In our study, we focused on male reproduction and how it can be affected by the ubiquitous presence of various substances. Furthermore, we compared four detection methods used to evaluate sperm capacitation status.

In the first part of our research, we examined the role of the natural hormone 17β-estradiol in sperm capacitation. This hormone is present in males under physiological conditions and regulates various cellular processes within the male reproductive tract. However, elevated estrogen levels lead to premature sperm capacitation in the cauda epididymis. Since successful egg fertilization relies on precise timing, alterations in sperm maturation may negatively impact reproductive fitness. This phenomenon may represent one of the pathways through which endocrine disruptors with estrogenic activity contribute to the increasing prevalence of fertility issues.

A standardized and reliable evaluation of capacitation, along with the selection of dependable detection methods, is essential for accurately assessing the fertilizing potential of individual sperm and sperm populations exposed to physiological or environmental factors. We demonstrated that all four methods tested can effectively detect the progress of capacitation. Furthermore, we highlighted their limitations and differences in detecting this progress. Both the CTC assay and the ACR.2 antibody assay can accurately predict the percentage of sperm undergoing acrosome reaction induced by the *zona pellucida*. However, the p-Y assay and FITC-Phall assay significantly underestimate this percentage. This discrepancy should be considered when investigating capacitation and the impact of different substances on both capacitation and acrosome reaction.

We also described the time sequence of molecular processes during capacitation, noting that initial observable changes include calcium redistribution and rearrangement of the acrosomal matrix, followed by increases in tyrosine phosphorylation and actin polymerization.

In the second part of our study, we demonstrated that even low concentrations of studied endocrine disruptors affect male reproduction at organ, cellular, and molecular levels. Using TBBPA as an example, we showed that exposure impact can be transmitted to subsequent generations, with continuous exposure across generations leading to an accumulation of negative effects. The study on zearalenone revealed that a low dose can have a greater impact on reproduction than a higher dose. Finally, the DEHP study indicated that exposure to

endocrine disruptors during critical developmental periods – such as masculinization programming window and puberty – may affect reproductive health in adulthood.

Alarmingly, the concentrations used in our studies correspond to real human exposure levels and are below established tolerable daily intake limits. Therefore, to accurately evaluate the true health risks associated with exposure to endocrine disruptors, it is essential to conduct studies using concentrations that reflect actual human and animal exposure levels.

Although we did not observe any effects on fertility in our studies, it is important to note that each substance was examined individually. In reality, individuals are exposed to hundreds or even thousands of different substances, many of which have confirmed or potential effects on reproduction. These substances may exert synergistic effects, compounding their impact on reproductive health.

Investigating endocrine disruptors is crucial for revealing their detrimental effects on both human and animal health, which may lead to the implementation of necessary legislative measures.

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Publication and author contributions

Doctoral thesis contains the following publications:

1. Ded L, Sebkova N, Cerna M, Elzeinova F, **Dostalova P**, Peknicova J, Dvorakova-Hortova K. **In vivo exposure to 17β-estradiol triggers premature sperm capacitation in cauda epididymis.** Reproduction. 2013 Mar 7;145(3):255-63. doi: 10.1530/REP-12-0472. Erratum in: Reproduction. 2015 Sep;150(3):X1. doi: 10.1530/REP-12-0472e. PMID: 23319664.

    - PD introduced the method for the detection of capacitation using CTC into laboratory practice.

2. Ded L, **Dostalova P**, Zatecka E, Dorosh A, Komrskova K, Peknicova J. **Fluorescent analysis of boar sperm capacitation process in vitro. Reprod Biol Endocrinol.** 2019 Dec 19;17(1):109. doi: 10.1186/s12958-019-0554-z. PMID: 31856844; PMCID: PMC6923987.

    - LD and PD contributed to the project equally. PD was responsible for investigation, methodology, data acquisition, and writing the manuscript.

3. 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. doi: 10.1016/j.reprotox.2012.05.095. Epub 2012 Jun 4. PMID: 22677475.

    - PD helped with the experiments and methodology and contributed to writing the manuscript.

4. 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. doi: 10.1016/j.reprotox.2014.01.003. Epub 2014 Jan 9. PMID: 24412631.

    - PD helped with the experiments and methodology and contributed to writing the manuscript.

5. **Dostalova P**, Zatecka E, Ded L, Elzeinova F, Valaskova E, Kubatova A, Korenkova V, Langerova L, Komrskova K, Peknicova J. **Gestational and pubertal exposure to low dose of di-(2-ethylhexyl) phthalate impairs sperm quality in adult mice.** Reprod Toxicol. 2020 Sep;96:175-184. doi: 10.1016/j.reprotox.2020.06.014. Epub 2020 Jun 30. PMID: 32619501.

    - PD participated in the study design, performed PCR and Western blot experiments, analyzed the results, wrote the original manuscript, and lead the revising process.

Publications not become a part of the doctoral thesis:

6. **Dostalova P**, Zatecka E, Dvorakova-Hortova K. Of Oestrogens and Sperm: **A Review of the Roles of Oestrogens and Oestrogen Receptors in Male Reproduction.** Int J Mol Sci. 2017 Apr 25;18(5):904. doi: 10.3390/ijms18050904. PMID: 28441342; PMCID: PMC5454817.

    - PD conducted an extensive literature review and utilized the findings from her research. She is the main author of the text and participated in the revision process.

7. 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. doi: 10.1186/1477-7827-8-87. PMID: 20626847; PMCID: PMC2908632.

    - PD prepared the media with specific concentrations of estrogens, performed sperm capacitation, and analyzed sperm capacitation status using CTC and the ACR2 antibody.

Supplements

Supplement 1

Ded L, Sebkova N, Cerna M, Elzeinova F, **Dostalova P**, Peknicova J, Dvorakova-Hortova K. **In vivo exposure to 17β-estradiol triggers premature sperm capacitation in cauda epididymis.** Reproduction. 2013 Mar 7;145(3):255-63. doi: 10.1530/REP-12-0472. Erratum in: Reproduction. 2015 Sep;150(3):X1. doi: 10.1530/REP-12-0472e. PMID: 23319664.

REPRODUCTION
RESEARCH

In vivo exposure to 17β-estradiol triggers premature sperm capacitation in cauda epididymis

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Abstract

Estrogens play a crucial role in spermatogenesis and estrogen receptor α knock-out male mice are infertile. It has been demonstrated that estrogens significantly increase the speed of capacitation *in vitro*; however this may lead to the reduction of reproductive potential due to the decreased ability of these sperm to undergo the acrosome reaction. To date the *in vivo* effect of estrogens on the ability of sperm to capacitate has not been investigated. Therefore, in this study, we exposed mice ($n=24$) to 17β-estradiol (E_2) at the concentration of 20 ng/ml either during puberty from the fourth to seventh week of age ($n=8$), or continuously from birth for a period of 12 weeks ($n=8$) at which age the animals from both groups were killed. The capacitation status of epididymal and testicular sperm was analysed by tyrosine phosphorylation (TyrP) antibody (immunofluorescence and western blot) and chlortetracycline (CTC) assay. According to our results, *in vivo* exposure to increased E_2 concentrations caused premature sperm capacitation in the epididymis. The effect of E_2, however, seems reversible because after the termination of the exposure premature epididymal sperm capacitation is decreased in animals treated during puberty. Furthermore the changes in epididymal sperm capacitation status detected by TyrP and CTC positively correlate with plasma levels of E_2 and the expression of the estrogen-dependent trefoil factor 1 (*Tff1*) gene in testicular tissue. Therefore, our data implicate that *in vivo* exposure to E_2 under specific conditions leads to the premature capacitation of mouse sperm in epididymis with a potential negative impact on the sperm reproductive fitness in the female reproductive tract.
Reproduction (2013) **145** 255–263

Introduction

Estrogens play a key role during male reproduction including spermatogenesis (Carreau *et al.* 2011, 2012) and sperm maturation such as capacitation (Baldi *et al.* 2009). Estrogen response is mediated through both genomic and non-genomic actions, where the genomic one involves binding to estrogen receptors ERs and nuclear transcription factors that activate the expression of target genes. The non-genomic estrogen action happens through transmembrane receptors such as ERA, ERB and G-protein coupled receptors (GPR30), triggering rapid intracellular signaling pathways, including the activation of serine/threonine and tyrosine phosphorylation (TyrP), leading to sperm capacitation (Kalab *et al.* 1998, Filardo *et al.* 2002). As a consequence of phosphorylation on mainly tyrosine residues, which is the key marker of successfully ongoing capacitation (Visconti *et al.* 1995*a*,*b*), the cytoskeleton protein network changes its dynamics, and acrosome reaction (AR) can occur (Yanagimachi 1994). Interestingly, progesterone-induced AR is inhibited by the extra amount of 17β-estradiol (E_2) through interaction with a specific non-genomic estrogen receptor on the sperm plasma membrane, suggesting that E_2, present at micromolar levels in follicular fluid, may act as a physiological modulator of sperm progesterone response, ensuring the appropriate timing of activation in the fertilisation process (Luconi *et al.* 1999). In rat and mouse males, the concentration of E_2 in blood plasma is 2–25 pg/ml, and it is lower than that in rete testis fluid (250 pg/ml; Free & Jaffe 1979). On the other hand, the concentration of estrogen in ovarian fluid is much higher when compared with the plasma (Free & Jaffe 1979, Hess *et al.* 1995) and it reaches multiple values depending on the time of the estrus fluctuating between 145 and 2100 pg/ml in rats and mice (Shaikh 1971). Most of our knowledge regarding these processes has been obtained from *in vitro* studies (Adeoya-Osiguwa *et al.* 2003, Ded *et al.* 2010, Sebkova *et al.* 2012), and for this reason it is really important to also do *in vivo* experiments.

Recent studies have shown that exposure to environmental estrogens may also influence male fertilising

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capability (Akingbemi 2005, Mathur & D'Cruz 2011). This includes chemicals that occur naturally in plants such as genistein and resveratrol (phytoestrogens) and also man-made chemicals such as bisphenol-A, diethylstilbestrol and vinclozolin (xenoestrogens), which can disrupt the endocrine function of animals and negatively influence spermatogenesis and sperm parameters (Peknicová *et al.* 2002, Kyselova *et al.* 2003, 2004, Elzeinova *et al.* 2008). These chemicals again mainly act by binding to estrogen receptors (Kuiper *et al.* 1998, Sohoni & Sumpter 1998, Akingbemi & Hardy 2001) and trigger a rapid non-genomic signaling response. The key estrogen receptor is ERA as its knock-out mice are infertile due to the disruption of the estrogen action within somatic cells of the reproductive system (Dupont *et al.* 2000). ERA activity has been demonstrated by expression of the trefoil factor 1 (*Tff1*) gene (Kim *et al.* 2000, Park *et al.* 2012), which is also a marker gene for the analysis of estrogenic activity (Dorosh *et al.* 2011).

The objective of this study was to test the *in vivo* effect of E_2 on the level of TyrP as a marker of ongoing mouse sperm capacitation and to monitor the *Tff1* gene expression as the marker for ERA activity.

Results

In vivo *exposure to E_2 increases the TyrP of epididymal sperm head proteins*

The number of positively labelled sperm heads for TyrP (Fig. 1) at the start of capacitation (time 0) was significantly higher in both experimental groups with pubertal (A) and continuous exposure to E_2 (B) compared with the control (Fig. 2). The difference between the control and experimental pubertal E_2 group was on the border of significance ($P \leq 0.05$). On the other hand, the difference between the control and experimental continuous E_2 group was highly significant ($P \leq 0.001$) despite the fact that the variance in the continuous E_2 samples was higher compared with the control and pubertal E_2 samples. Moreover, the difference between pubertal E_2 and continuous E_2 was also significant ($P \leq 0.01$), thus indicating that sperm cells from animals after continual exposure to E_2 (continuous E_2) are highly different to those after pubertal exposure (pubertal E_2) and the control.

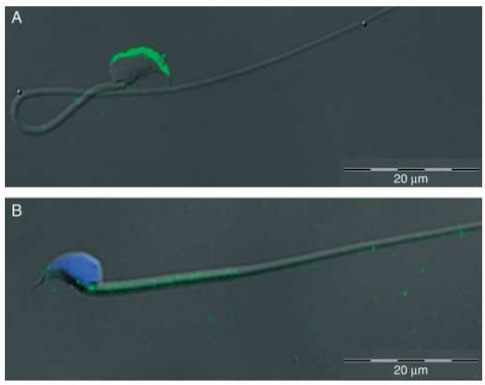


Figure 1 Immunoflourescent detection of tyrosine phosphorylation (TyrP) in mouse epididymal sperm. (A) TyrP-positive sperm head and (B) TyrP-negative sperm head. Scale bar 20 µm.

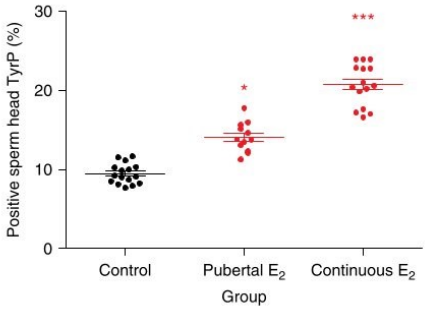


Figure 2 Number of capacitated epidymidal sperm detected by anti pY antibody (time 0). Control group, pubertal E_2 and continuous E_2. Bars denote arithmetical means of capacitated cells (%), whiskers denote S.E.M. and points denote individual measurements. *$P<0.05$, ***$P<0.001$.

In vivo *exposure to E_2 changes the distribution of chlortetracycline fluorescent patterns in epididymal sperm cells*

In addition to the analysis of the sperm head TyrP, chlortetracycline (CTC) analysis was performed to evaluate changes in CTC fluorescent patterns relating to sperm capacitation (Fig. 3). Data from the CTC analysis correlate with the data from TyrP analysis. The control group had the lowest number of cells with CTC pattern B relating to the capacitated status of the sperm (Fig. 3). Similar to the data from TyrP analysis, the number of cells with CTC pattern B (Fig. 3) relating to the capacitated status was significantly higher in both experimental groups compared with the control (Fig. 4). The difference between the control and pubertal E_2 was the lowest ($P \leq 0.05$), and the difference between the control and continuous E_2 was the highest ($P \leq 0.001$). Also the difference between the two experimental groups, pubertal E_2 and continuous E_2, was significant. This fact indicates the significant changes in the distribution of the CTC fluorescent patterns after the termination of E_2 exposure in the pubertal E_2 group. Together, with the data from TyrP labelling, western blot (WB; Fig. 5) and CTC analyses provide strong evidence for the procapacitation effect of E_2 based on the analysis of these two parameters. Also the correlation between the number of TyrP-positive sperm heads and the number of spermatozoa with the pattern B was high ($r=6.683$, $P=0.0358$).

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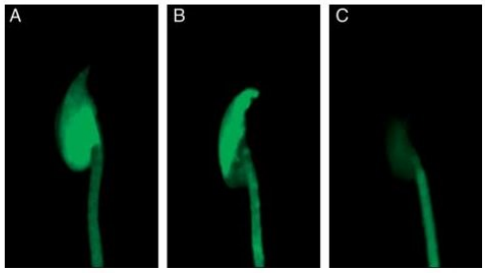


Figure 3 Patterns of fluorescence in CTC staining. (A) Bright fluorescence over the entire head with a brighter line of fluorescence across the equatorial segment (pattern F, non-capacitated spermatozoon). (B) Bright fluorescence in the anterior segment of the head with an absence of fluorescent labelling over the equatorial and post-equatorial segments (pattern B, capacitated spermatozoon). (C) Low or absent fluorescence over the entire head (pattern AR, acrosome-reacted spermatozoon).

The initial differences of the sperm head TyrP are propagated during in vitro *capacitation*

After analysis of the native epididymal sperm suspension, sperm were capacitated to assess the potential differences in the capacitation progress at individual capacitation times. At the start of the capacitation process, there exist significant differences between the control and experimental groups representing the differences in the phosphorylation of the sperm head proteins directly in the epididymis (Fig. 6). These differences were propagated into the subsequent capacitation times. After 30, 60, 90 and 120 min of capacitation, the differences between the control and experimental groups followed those from the time 'zero' representing a positive epididymal sperm head TyrP. The highest, but not statistically significant, difference between the capacitation progress was at 30 min of capacitation (time) for continuous E_2 (Fig. 7). This difference indicates the fact that some population of the spermatozoa from animals exposed to E_2 is on the border of the capacitation status and after the initial capacitation impulse, this population responds by increasing the total amount of capacitated spermatozoa in the first subsequent capacitation time (30 min; Fig. 7). The data from positive TyrP labelling were further supported by WB analysis of TyrP (Fig. 5). The difference between the control and pubertal E_2 is not visible at the time 0. On the other hand, there is a higher TyrP of proteins around 68 and 45 kDa for continuous E_2. Therefore, the evidence from WB partially supports data from TyrP fluorescence, despite the fact that WB also included differences between TyrP of the tail-associated proteins, which were not considered during the TyrP fluorescence analysis.

The indicators of capacitation status positively correlate with the serum levels of E_2 and expression of Tff1 *gene*

For the analysis of the connection between the indicators of sperm capacitation status and other relevant physiological parameters of estrogen action, the measurement of E_2 serum levels (Fig. 8A) and expression of the estrogen-dependent *Tff1* gene (Fig. 8B) were analysed and statistically correlated with the previous results from the sperm analysis. According to the obtained results, the percentage of the TyrP-positive sperm head cells in the epididymis positively correlates both with E_2 plasma levels and with the expression of the *Tff1* gene in the testicular tissue. The concentration of plasma E_2 in the control group and pubertal E_2 group was relatively similar with the arithmetical mean around 46 pg/ml and had a S.D. of 17. On the other hand, plasma E_2 levels in Continuous E_2 group were much higher and reached an average concentration of 3705 pg/ml with an S.D. of 205. The correlation coefficient r between the number of TyrP-positive sperm cells and plasma levels of E_2 was 0.549 ($P \leq 0.05$), which indicates a high correlation between these two parameters (Fig. 8A). Also the correlation between the expression of the *Tff1* gene expressed as C_q values and the number of TyrP-positive sperm were high ($r = -0.881$, $P \leq 0.001$) indicating a strong connection between these two parameters (Fig. 8B).

The TyrP of testicular epididymal sperm head proteins is not altered after in vivo *exposure to E_2*

To examine the potential effect of E_2 exposure on the capacitation status of testicular sperm, the TyrP of epididymal sperm head proteins was analysed (Fig. 9). In the control and both experimental groups, the percentage of TyrP-positive sperm heads was relatively low (about 1.5%) with no significant differences between

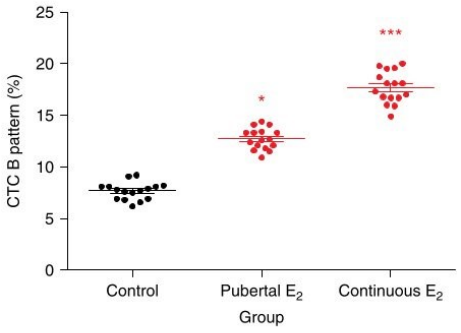


Figure 4 Number of capacitated epidymidal spermatozoa detected by CTC staining (time 0). Control group, pubertal E_2 and continuous E_2. Bars denote arithmetical means of capacitated cells (%), whiskers denote S.E.M. and points denote individual measurements. $^*P<0.05$, $^{***}P<0.001$.

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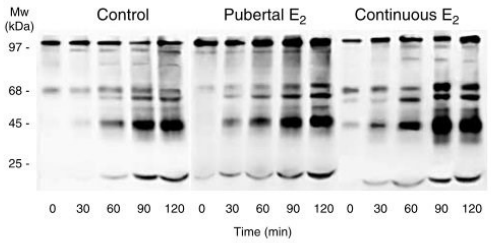


Figure 5 Western blot analysis of protein phosphorylation during *in vitro* capacitation detected by anti pY antibody. Control group (C), pubertal E_2 and continuous E_2. The higher phosphorylation of proteins at the start of capacitation (bands around 68 and 45 kDa) in continuous E_2 is visible. The highest cumulative protein phosphorylation in subsequent capacitation intervals is visible.

control and experimental groups and also without any significant difference between the two experimental groups ($P \geq 0.05$; Fig. 10). Owing to the fact that no significant differences among the groups were observed, the power analysis for the statistical test was performed and it was 0.227.

Discussion

Estrogens play an important role in almost all sperm physiological processes from the production of sperm in the testes to fertilisation in the female oviduct. For the last decade, the effect of estrogens on specific physiological processes in sperm has largely been studied during *in vitro* experiments. In these experiments, natural and synthetic estrogens have shown a procapacitation concentration-dependent effect on epididymal (mouse; Sebkova *et al.* 2012) and ejaculated spermatozoa (boar; Ded *et al.* 2010). Exposure to estrogens during *in vitro* capacitation provides a good model for simulating the *in vivo* conditions in the female reproductive tract. On the other hand, sperm are exposed to estrogens and various compounds with estrogenic activity from the time of their production to their release into the testicular tubule. Owing to the fact that some of the sperm already express specific indicators of the capacitation status in the epididymis, it is important to know whether or not the sperm capacitation status can be altered during sperm storage in the cauda epididymis in response to the estrogen stimuli. This type of study required *in vivo* exposure to estrogens; therefore, the most common and well-studied E_2 was selected for this purpose. We have shown that molecular capacitation markers in sperm after *in vivo* exposure to E_2 are similar to those observed *in vitro*. Both TyrP and CTC pattern distribution were changed in a similar way after *in vitro* and *in vivo* exposure.

Contrary to previous *in vitro* experiments, during *in vivo* exposure, E_2 does not reach sperm cells directly, but it has to pass through multiple biological barriers before it is able to have a possible effect on epididymal sperm. The normal serum concentration of E_2 is at the range of 2–20 pg/ml (Snyder *et al.* 2009). There is also evidence that in the rete testis the concentration of estrogens is tenfold higher and related to the plasma levels (Free & Jaffe 1979). In our study, the extensiveness of the E_2 effect on the sperm physiological parameters positively correlates with the serum levels. We also measured the expression of the *Tff1* gene, the specific estrogen response gene in the testicular tissue. The correlation between the expression of this gene and physiological parameters of the spermatozoa was also positive and more significant compared with the plasma levels of E_2. Therefore, the extensiveness of the molecular changes in epididymal sperm cells is largely dependent on the plasma and tissue levels of E_2. After exposure the phosphorylation status and CTC patterns relating to calcium homeostasis then returned to levels more similar to the control ones, which may show that the effect can be reversible if the exposure is terminated. Despite this process, there are still differences between the control and experimental pubertal E_2 groups in the number of cells with capacitated patterns. This situation may be related to the fact that although there are no differences in the plasma concentration of estrogens the concentration in the reproductive tract could be still higher. The higher expression of *Tff1* gene in pubertal E_2 supports this hypothesis. On the other hand, the molecular changes initiated by estrogens on the level of spermatogenesis and propagated into the subsequent developmental changes of the sperm cannot be excluded, as the gene expression pattern is altered weeks after the exposure and could be propagated by epigenetic mechanisms to subsequent generations.

During *in vivo* exposure the estrogens can also influence the future sperm capacitation ability during

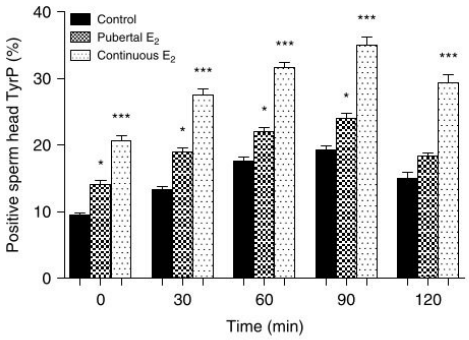


Figure 6 Number of capacitated spermatozoa after 0, 30, 60, 90 and 120 min of *in vitro* capacitation. Capacitation progress was measured by anti pY antibody (Fig. 1). $*P<0.05$, $***P<0.001$.

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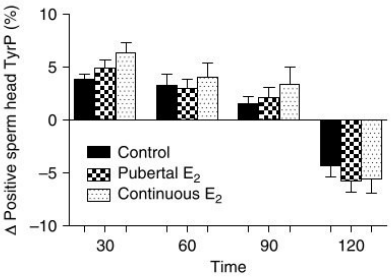


Figure 7 Differences (Δ) in the number of capacitated spermatozoa detected by anti pY antibody between two subsequent capacitation times (30–0, 60–30, 90–60, 120–90) in individual control and experimental groups.

spermatogenesis and sperm storage. Therefore, the analysis of the capacitation status of testicular sperm has been performed in the current study. During this analysis, there were no significant differences in the number of TyrP-positive testicular spermatozoa among the control and experimental groups. Based on the results of a previous *in vitro* study (Sebkova *et al.* 2012), it can be assumed that with an elevated *in vivo* estrogen exposure, capacitation may start in the epididymal spermatozoa. On the other hand, it has been reported that estrogens trigger multiple phosphorylation pathways in the testicular tissue or can change their cell sensitivity (Miyaso *et al.* 2012). For this reason it is possible to consider that potential molecular changes in testicular sperm, and individual sperm developmental stages can lead to a higher number of capacitated sperm observed in the epididymis. As shown in this study the sensitivity (statistical power) of testicular sperm head TyrP analysis was lower compared with the epididymal one and therefore the potential differences in this parameter in testicular tissue cannot be fully excluded.

The premature molecular changes related to the capacitation process may lead to many consequences after ejaculation and physiological capacitation in the female reproductive tract. The precise timing of individual molecular processes during the capacitation is an absolute prerequisite for successful fertilisation. From the population of millions of sperm there are only tens or hundreds reaching the egg and only one can successfully fertilise it. Already at the start of capacitation, sperm cells are unequal concerning their morphology and ability to reach, penetrate and fertilise the egg. It has been recently shown that higher TyrP can lead to a decreased ability of sperm to undergo AR in the presence of calcium-ionophore (Sebkova *et al.* 2012). The hyperphosphorylation of sperm proteins may therefore lead to the inability of sperm to undergo AR.

On the other hand, calcium changes detected by the CTC method are prerequisite for physiological AR and egg fertilisation. In general, considering a wide range of mammalian species, including humans, the premature calcium influx induced by estrogens may lead to premature AR without the presence of zona pellucida factors. Together, with the hyperphosphorylation of sperm proteins the percentage of spermatozoa with an ideal physiological status relating to their ability to reach and fertilise the egg strongly decreases.

The population of epididymal spermatozoa is different concerning its maturation and 'capacitation' status. This diversity seems to be a good criterion for successful fertilisation, due to the high number of spermatozoa, as it is usual in each sperm population that there is a subpopulation of spermatozoa with ideal molecular characteristics to fertilise the egg. Estrogens and other

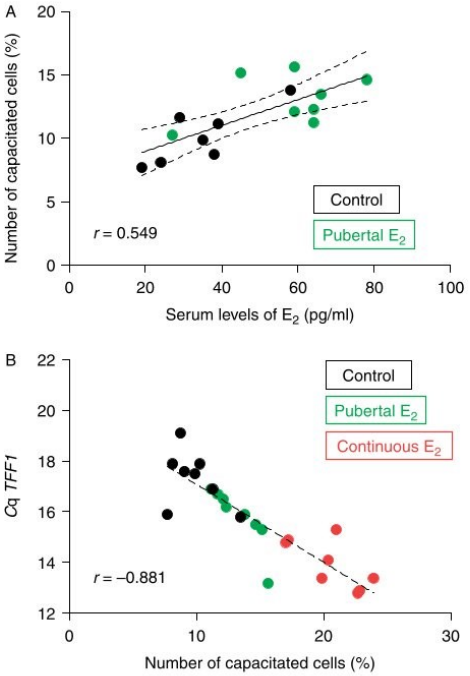


Figure 8 (A) The correlation between the number of capacitated epidymidal sperm (time 0 of the capacitation) detected by anti pY antibody and E_2 plasma levels detected by RIA in pubertal E_2 and control groups. Each data point represents the measurement of both parameters from one animal. Individual groups are labelled with different colours. The highly different continuous E_2 group was excluded from this analysis. The straight line indicates the Pearson coefficient (r), the dotted lines indicate (95%) confidence intervals. (B) The correlation between the number of capacitated epidymidal spermatozoa (time 0 of the capacitation) detected by anti pY antibody and the expression of the estrogen-dependent *Tff1* gene. The expression of the *Tff1* gene is expressed as the normalised C_q values. Each data point represents the measurement of both parameters from one animal. Individual groups are labelled with different colours. The dotted line indicates the Pearson coefficient (r).

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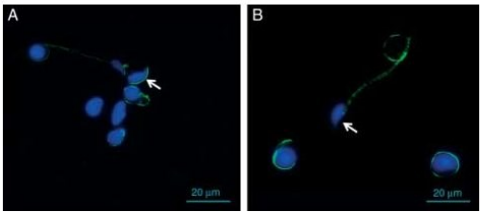


Figure 9 Immunofluorescent detection of TyrP in testicular sperm head. (A) Positive testicular sperm head (indicated by an arrow), (B) negative testicular sperm head. Scale bar 20 μm.

compounds with estrogenic activity can affect the balance of the capacitation status directly in the epididymis and through this activity they may decrease the fertilising potential of spermatozoa. After terminating the E_2 exposure, the situation more or less went back to normal, which was close to the status of the control group; therefore, this effect seems to be reversible. Based on our data, it is also clear that *in vivo* E_2 exposure influences the proportion of spermatozoa with the specific molecular characteristics relating to only the beginning of the capacitation. During the subsequent *in vitro* capacitation, the differences between the control and experimental groups remained the same as observed at the beginning. It implies that the sperm population, of the exposed animals reaching the egg could have a significantly altered capacitation status and a lower fertilising potential compared with the control ones.

The premature sperm capacitation in the cauda epididymis may be one of the factors bringing a negative effect of the endocrine-disrupting chemicals on the male reproductive functions. It has been recently shown that not only natural but also artificial estrogens significantly affect the sperm capacitation process (Sebkova *et al.* 2012). Although 17α-ethynylestradiol only seems to act at higher concentrations ($\geq$2 ng/ml), the combination of all substances with potential or confirmed estrogenic activity can further misbalance the physiology of epididymal spermatozoa. Furthermore some artificial compounds with estrogenic activity have a significant effect on sperm physiology at lower doses compared with the strongest natural estrogen such as E_2 (Fraser *et al.* 2006, Sebkova *et al.* 2012).

In conclusion, our data imply that *in vivo* exposure to E_2 leads to premature 'capacitation' of mouse sperm in the cauda epididymis with a further potential negative impact on sperm reproductive fitness in the female reproductive tract. This effect is caused mainly by the hyperphosphorylation of sperm proteins and a/the premature calcium influx. These processes lead to a decreased ability of sperm to undergo an AR. Based on this evidence, *in vivo* exposure to E_2 can lead to a decrease of the fertilising potential in male mice with significant relevance to endocrine-disrupting compounds with estrogenic activity.

Materials and Methods

Animals and E_2 exposure protocol

Inbred mice (Anlab, Prague, Czech Republic) were housed in an animal facility (Institute of Molecular Genetics, ASCR, v. v. i.). Estrogen-free food and water were supplied *ad libitum*. The total number of animals in the experiment was 24 in each group, sperm from both epididymides (n=16) of eight animals were analysed (n=8). Male mice were exposed to E_2 (Sigma) at a 20 ng/ml concentration either during puberty from of age (Pubertal E_2) or continuously from birth to 12 weeks of age (Continuous E_2). The control group of mice was not exposed to E_2. At the age of 12 weeks, the animals from all groups were killed by cervical dislocation. All animal procedures were carried out in strict accordance with the Animal Scientific Procedure, Art 2010, and approved by the Local Ethics Committee (approval number 151/2009).

Sperm preparation and capacitation

Mouse sperm cells were released from the distal regions of cauda epididymis into M2 fertilising medium (Sigma) under paraffin oil at 37 °C in 5% CO_2. Sperm viability and motility were checked (under a light microscope). Sperm stock was diluted to the required concentration (5×10^6/ml) into M2 medium under paraffin oil. Sperm samples were collected at 0, 30, 60, 90 and 120 min of capacitation *in vitro*. The time marked as 0 was the minimum time required for sperm to be added to the capacitated medium, removed from the medium and washed. This manipulation did not exceed 1 min. These samples served as a negative control. Sperm motility and viability were controlled at every experimental time point.

Immunofluorescent detection of TyrP

Samples of sperm cells were spread on microscope slides. After air-drying, sperm were fixed with 3.7% formaldehyde in PBS (pH 7.34) at room temperature for 10 min, followed by washing in PBS, incubated with 15 mM ammonium chloride (NH_4Cl)

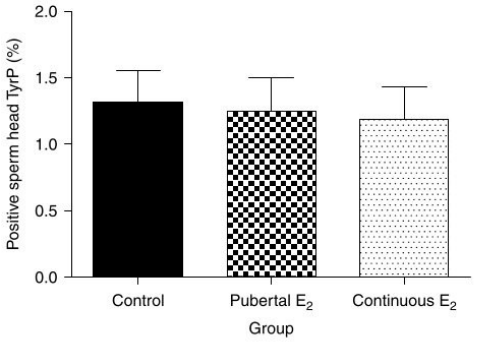


Figure 10 Number of TyrP-positive testicular sperm heads (%) among individual groups. Bars denote the arithmetical mean, whiskers denote S.E.M.

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for 5 min, and with 0.1% Triton X-100 for 3 min. Slides were washed with PBS which was followed by immunofluorescent staining. Sperm were blocked with 10% BSA in PBS for 1 h and incubated with primary MAB anti-phosphotyrosine P-Tyr-01 (Exbio, Prague, Czech Republic) diluted 1:500 in 1% BSA in PBS over night at 4 °C, followed by Alexa Fluor 488 donkey anti-mouse IgG (Molecular Probes, Prague, Czech Republic) secondary antibody 1:1000 in PBS for 1 h. Slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Slides were examined with an epifluorescent microscope. For every experiment, we collected sperm data from eight mice. The positive or negative signal was evaluated from a total of 200 spermatozoa on every slide. In each group, at least two samples were analysed. Data were analysed statistically.

CTC fluorescent assay

Spermatozoa were resuspended in PBS and mixed with an equal volume (45/45 µl) of CTC solution (750 mmol/l CTC in 130 mmol/l NaCl, 5 mmol/l cysteine, 20 mmol/l Tris–HCl, pH 7.8) and incubated for 30 min. Cells were then fixed with 8 µl of 12.5% paraformaldehyde in 0.5 mol/l Tris–HCl (pH 7.4). After incubation, sperm suspension was smeared onto a glass slide and covered with a cover slip. To avoid evaporation and CTC fading, slides were kept in a wet chamber until evaluation. Samples were examined with a Nikon Labothot-2 fluorescent microscope equipped with a 40× Nikon Plan 40/0.65 and photographed with a COHU 4910 CCD camera (COHU Inc. Electronics Division, San Diego, USA) with LUCIA imaging software (Laboratory Imaging Ltd., Prague, Czech Republic).

SDS–PAGE with immunoblotting

SDS electrophoresis and immunoblotting technique was used for the TyrP assessment and was carried out using protocols based on standard methods (Laemmli 1970, Zigo *et al.* 2011). Suspension of non-capacitated sperm from a sperm stock released from cauda epididymis was used. Sperm samples were collected at 0, 30, 60, 90 and 120 min of capacitation *in vitro*, diluted with PBS and a final concentration of 10^6 sperm cells was ascertained using a Bürker chamber. Sperm pellets were resuspended in an equal volume of SDS–PAGE non-reduced sample buffer and heated at 97 °C for 3 min. Samples containing protein equivalent to 10^6 capacitated sperm cells were run on a 5% stacking and 10% running SDS polyacrylamide gel using Precision Plus Protein All Blue standards (Bio-Rad) as molecular weight markers. After transferring proteins onto a nitrocellulose membrane, non-specific sites were blocked with PBS blocking solution (5% skimmed milk and 0.05% Tween 20). Proteins phosphorylated on tyrosine residues were identified by the primary MAB anti-phosphotyrosine P-Tyr-01 (Exbio) diluted 1:500, followed by a peroxidase goat anti-mouse IgG secondary antibody (Sigma–Aldrich) diluted 1:20 000. Protein staining was visualised by chemiluminescence Super Signal West Dura (Thermo Scientific, Prague, Czech Republic). These experiments were performed at least three times with similar results. Representative results are shown.

Gel densitometry was performed with an Aida image analyser 4.18 (Raytest GmbH, Sprockhövel, Germany) and the relative intensity of individual signals was determined.

Quantitative RT-PCR (RTqPCR) of Tff1 *gene*

The analysis was performed according to the protocol from our previous study (Zatecka *et al.* 2013). The total RNA from one testis per animal was extracted by a Tri-Reagent Kit (Sigma) according to the manufacturer's instructions. Isolated RNA was stored at −70 °C. The RNA quality and purity was measured on a spectrophotometer Helios A (Thermo Electron Corporation, Marietta, OH, USA). For the synthesis of cDNA, 5 µg purified RNA was used. Each sample was treated with 1 µl DNase I (Invitrogen), 1 µl DNase I reaction buffer (Fermentas, Burlington, ON, 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, NJ, USA). After incubation 1 µl EDTA (Fermentas) was added and followed by incubation at 65 °C for 10 min. Then 30 µl reaction mixture (8 µl reaction buffer for M-MuLV reverse transcriptase (Fermentas), 5 µl 10 mM 4dNTP (Fermentas), 0.3 µl RiboLock inhibitor (Fermentas), 1 µl oligo (dT)+random primers (Promega) and 15.2 µl H_2O were added to the samples. The mixture was incubated for 60 min at 42 °C followed by 10 min at 70 °C and at the end was maintained at 4 °C. Obtained cDNA was stored at −20 °C. For RT-qPCR – 5× diluted cDNA was used. The qPCR primer pair 5′-TGTCCGGGGATTCCCGTGGT-3′ (forward) and 5′-CCAGTGCCCAGGTGGAGGGT-3′ (reverse) specific for the mouse *Tff1* gene was used for RT-qPCR. The product length was 131 bp. For each reaction, 2 µl 5× diluted cDNA, 10 µl SYBR Green Master Mix (Fermentas), 0.5 µl primer and 7 µl H_2O were used. All reactions were performed in duplicates in a PCR cycler (Eppendorf, Prague, Czech Republic). The relative amount of mRNA in each sample was calculated from the measured quantification cycle (C_q) values. The expression of the reference gene for ribosomal 18S was used to normalise the measured values. The qPCR primer pair 5′-GTAACCCGTT-GAACCCCATT-3′ (forward) and 5′-CCATCCAATCGGTAG-TAGCG-3′ (reverse) with the product length 151 bp was used.

Measurement of the E_2 serum levels

Whole blood from mice was left for 60 min at room temperature. After incubation, the blood samples were centrifuged at 1200 ***g*** for 15 min. Serum levels of E_2 were analyzed using a RIA kit (Immunorad Beckman, Prague, Czech Republic). The detection limit was <2 pg/ml and the cross reactivity of antiserum with other serum estrogens was lower than 1%.

Preparation and analysis of testicular suspensions

Testes were placed into a glass homogeniser with 2 ml PBS and homogenised manually to obtain single-cell testicular suspension. After the homogenisation procedure the suspension was filtrated by a Cell Strainer 70 µm (BD Bioscience, Prague, Czech Republic) to remove residues of tough tissue. After

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filtration, single-cell testicular suspension was centrifuged (300 ***g***), resuspended in 1 ml 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. Samples of testicular suspensions were resuspended and washed 2× in PBS and spread on microscope slides. After this procedure, the protocol was the same as for the immunofluorescent detection of TyrP on epididymal sperm, but only 100 cells per slide were counted.

Statistical analysis

Experimental data were analysed using STATISTICA 6.0 (Statsoft, Prague, Czech Republic) and GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA, USA). The differences between the control and experimental groups in the number of the capacitated cells (TyrP positive sperm heads and CTC pattern B, Figs 2, 4, 6 and 7) were analysed by KW-ANOVA, and *post hoc* analysis was performed by Dunn's comparisons: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. The calculated Pearson coefficients (r) were tested for their significance ($P\leq0.05$, Figs 8 and 9). The statistical power of the testicular sperm analysis was performed by STATISTICA 6.0 power module and was calculated for the ANOVA test.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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METHODOLOGY Open Access

Fluorescent analysis of boar sperm capacitation process in vitro

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Abstract

Background: Capacitation involves physiological changes that spermatozoa must undergo in the female reproductive tract or in vitro to obtain the ability to bind, penetrate and fertilize the egg. Up to date, several methods have been developed to characterize this complex biological process. The goal of the presented study is to mutually compare several fluorescent techniques, check their ability to detect changes in molecular processes during the capacitation progress and determine their ability to predict the percentage of acrosome reacted (AR) sperm after the exposure to solubilized *zona pellucida* (*ZP*). The capacitation process was analyzed using four fluorescent techniques: 1. chlortetracycline (CTC) staining, 2. anti-acrosin antibody (ACR.2) assay, 3. anti-phosphotyrosine (pY) antibody assay, 4. fluorescein isothiocyanate-conjugated phalloidin (FITC-phall) assay. All these methods were tested using fluorescent microscopy and flow cytometry.

Results: All selected methods are capable to detect the capacitation progress of boar sperm in vitro, but there are significant differences in their outcome when using fluorescent microscopy or flow cytometry experimental arrangements and subsequent statistical analysis (KW-ANOVA). Also, the ability to predict the absolute numbers of sperm which will undergo *ZP*-induced AR differ significantly (CTC and ACR.2 gave the best predictions).

Conclusions: Our study compared four largely used methods used to characterize capacitation process, highlighted their differences and showed that all are able to detect capacitation progress, CTC and ACR.2 are furthermore able to accurately predict the percentage of AR sperm after *ZP*-induced AR.

Keywords: Acrosome reaction, Fluorescent microscopy, Flow cytometry, Chlortetracycline assay, Tyrosine phosphorylation, Acrosin staining, Phalloidin staining

Introduction

Capacitation is a physiological process that spermatozoa must experience in the female reproductive tract or in vitro to obtain the ability to bind, penetrate and fertilize the egg [1–3]. The capacitation is based on many molecular processes including changes in the intracellular calcium concentration [4], rearrangement of the acrosomal matrix [5], rearrangement of the sperm cytoskeleton [6–8], phosphorylation of sperm proteins [9, 10] and changes in the sperm plasma membrane [11].

Since the discovery of capacitation, several methods have been developed to characterize this complex biological process. There are four major fluorescent methods, to be mentioned, and they all target different sperm characteristics: 1. CTC method detects the redistribution of the intracellular calcium in the sperm head during capacitation [12, 13]; 2. ACR.2 recognizes the rearrangement of the acrosomal matrix by detecting changes in the accessibility of acrosin epitopes. The higher accessibility of the acrosin epitopes is a significant marker of capacitation progress [14]; 3. FITC-phall) binds to F-actin, as actin polymerization significantly increases during capacitation progress [15]; 4. Fluorescein isothiocyanate-conjugated antibodies, such as anti-phosphotyrosine (pY) antibody (anti-pY), detecting a capacitation dependent phosphorylation of various proteins [16, 17].

All accounted methods can be used in various experimental protocols, e.g. CTC in fluorimetry, ACR.2 in ELISA, anti-Y in western blot etc. Fluorescent analysis is a general method suitable for all detection procedures and generally, there are two ways to perform fluorescent

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analysis on a cellular level: 1. by fluorescent microscopy and 2. flow cytometry. The physiological acrosome reaction (AR) is triggered by glycolytic extracellular matrix of the egg called *zona pellucida* (*ZP*) [18].

A standardize and reliable evaluation of capacitation and the selection of a reliable detection methods is a methodological prerequisite for the quality assessment of fertilizing potential of individual sperm and sperm population exposed to physiological or environmental factors. In our study, we focused in detailed on analyzing the capacitation process of boar sperm through fluorescent detection using both fluorescent microscopy and flow cytometry. The aim of this work was to assess the ability of individual methods to detect relevant molecular changes during sperm capacitation; to compare their advantages and disadvantages in order to select a suitable method for evaluation of sperm capacitation and estimate the potential of individual methods to predict sperm ability to undergo *ZP* triggered AR and subsequently fertilize the oocyte.

Materials and methods

Chemicals

All chemicals were purchased from Sigma (Prague, Czech Republic) unless otherwise specified.

Sperm preparation, capacitation in vitro and zona pellucida-induced acrosome reaction

Boar (*Sus scrofa*) ejaculates (20 ejaculates from 20 individual animals) were supplied by Insemination Station, Kout na Sumave, CR as chilled (17 °C) and diluted samples [19]. All sperm samples were examined for motility and viability (minimal parameters to include a sample into the analysis were 80% motility, 80% viability; the actual variability of both parameters was not higher than 5% among all samples included to the analysis), washed twice in tris-buffered saline (TBS, 200 x g, 10 min), centrifuged on Percoll gradient (80, 70, 55, 40% Percoll, 200 x g, 60 min) and washed in capacitation medium without bovine serum albumine (11.3 nM NaCl, 0,3 mM KCl, 1 mM $CaCl_2$, 2 mM TRIS, 1.1 mM glucose, 0.5 mM pyruvate). Sperm were resuspended in capacitation medium containing BSA (1 mg/mL) to concentration 5×10^7 sperm/ml. and suspension was incubated for 60, 120, 180, 240 min under paraffin oil at 37 °C, 5% CO_2.

After 240 min of incubation, selected samples incubated for 240 min were treated by boar solubilized *ZP* (Czech University of Life Sciences, Prague, Czech Republic) for 60 min (37 °C, 5% CO_2) [18] to induce acrosome reaction. The percentage of acrosome reacted sperm was determined by staining the acrosomes with FITC-conjugated *Pisum sativum* agglutinin (PSA).

CTC and indirect immunofluorescence assays

The CTC was performed as described previously [13] using the following protocol. After the capacitation process (60, 120, 180, 240 min) sperm suspensions were centrifuged at 200 x g, for 5 min; the capacitation medium was removed and kept at − 20 °C. Sperm were re-suspended in phosphate-buffered saline (PBS) and mixed with equal volume (45 μl/45 μl) of CTC solution (750 mmol/l CTC in 130 mmol/l NaCl, 5 mmol/l cysteine, 20 mmol/l Tris-HCl, pH 7.8) and incubated for 30 min. Cells were then fixed in 8 μl of 12.5% paraformaldehyde in 0.5 mol/l Tris-HCl (pH 7.4). After incubation, sperm suspension was smeared onto a glass slide covered by a cover slip. To avoid evaporation and CTC fading, slides were kept in a dark wet chamber and immediately evaluated.

ACR.2 (Exbio 11–260-C100) immunofluorescent analysis was described previously [20]. After the capacitation process, sperm suspensions from all incubation times (60, 120, 180, 240 min) were centrifuged (200 x g, 5 min); the capacitation medium was removed, and kept at − 20 °C. Sperm were re-suspended in equal volume of phosphate-buffered saline (PBS), smeared onto glass slides, dried and kept at 4 °C. During fluorescent labelling preparation, sperm slides were fixed with acetone for 10 min, rinsed with PBS, treated with ACR.2 monoclonal antibody (50 μg/ml), anti-pY antibody (Sigma-Aldrich P5872; 10 μg/ml) or FITC-phall (Sigma-Aldrich P5282; 50 μg/ml) binding specifically to actin filaments, and incubated in a wet chamber for 60 min at 37 °C. After thorough washing in PBS, the ACR.2 and anti-pY smears were treated with FITC-conjugated anti-mouse IgG antibody (Sigma-Aldrich F0257; 1:500) and incubated in a wet chamber for 60 min at 37 °C. After washing in PBS and water, smears were mounted by the Vectashield mounting medium with DAPI (Vector Lab., Burlingame, CA).

Samples were examined with a Nikon Labothot-2 fluorescent microscope equipped with 40x Nikon Plan 40/0.65 and photographed with a COHU 4910 CCD camera (Inc. Electronics Division, San Diego, USA) using LUCIA imaging software (Laboratory Imaging Ltd., Prague, Czech Republic). Sperm cells were classified according to their cellular (acrosomal) staining patterns into non-capacitated, acrosome intact sperm; capacitated, acrosome-intact sperm; and acrosome-reacted sperm (Table 1; Fig. 1). In each sample, 200 cells were evaluated.

Flow cytometry analysis

Sperm samples were collected at different time during capacitation process (0, 60, 120, 180, 240), then centrifuged and washed in PBS (200 x g, 5 min) and fixed by 96% ethanol at 4 °C for 30 min. After ethanol fixation, sperm were re-fixed in ethanol-acetone mixture at 4 °C

Table 1 Specific fluorescent patterns of the boar sperm (chilled 17 °C/diluted) as detected by individual fluorescent methods

Assay	chlortetracycline (CTC)	anti-acrosin antibody (ACR.2)	anti-phosphotyrosine (pY)	fluorescein isothiocyanate-conjugated phalloidin (FITC-phall)
Non-capacitated, acrosome intact sperm	Bright fluorescence over the entire sperm head and positive mid-piece of the tail	Moderate fluorescence in the acrosome	Low fluorescence in the sperm head, triangular segment within the head and tail	Moderate fluorescence in the sperm head and tail
Capacitated, acrosome-intact sperm	Prominent fluorescence in the equatorial segment, mid-piece of the tail and no fluorescence (dark) in the post-acrosomal region of the head	Intensive fluorescence in the acrosome	Intensive fluorescence in the sperm head, triangular segment within the head and tail	Intensive fluorescence in the acrosome and tail
Acrosome-reacted sperm	Low fluorescence over the sperm head, with remaining positive signal in the equatorial segment and mid-piece of the tail	Low or no fluorescence in the sperm head with a remaining fluorescence in the equatorial segment of the sperm head	No fluorescence in the acrosome with a remaining positive fluorescence in the triangular segment within the head and tail	Low fluorescence in the sperm head with a remaining positive signal in the tail

(10:1) for 30 min. CTC treatment was carried out as described previously. Sperm intended for other analysis were washed three times in PBS and incubated with anti-acrosin ACR.2 antibody (50 μg/ml), anti-pY antibody (Sigma-Aldrich P5872; 5 μg/ml) and FITC-phall (Sigma-Aldrich P5282; 10 μg/ml) at 37 °C for 60 min. After the incubation with the primary antibody (ACR.2, anti-pY), sperm were washed three times in PBS and incubated with a FITC-conjugated anti-mouse IgG antibody (Sigma-Aldrich F0257; 1:1000)) for 60 min. FITC-phall samples were only kept in incubation chamber. After the incubation, all sperm samples were intensively washed in PBS (five times for 5 min) and subsequently 100 μl of the suspension was placed on 96-well plate. Flow cytometry data acquisition was performed on BD LSR II instrument (BD, Becton Drive Franklin Lakes, NJ, USA), excitation laser 488 nm, emission filters 530/40, measurement of fluorescent intensity in FITC channel. Analysis was performed using FlowJo 7.5.4. software (TreeStar Inc., Ashland, OR, USA; Additional file 2: Figure S2). The differences among individual samples in the % of cells in appropriate gates (NC – non-capacitated, C - capacitated, AR – after acrosome reaction) and arithmetic mean of the fluorescent intensity in the FITC channel (CTC) were assessed.

Statistical analysis

Experimental data were analyzed using STATISTICA 7.0. (StatSoft CR, Prague, Czech Republic) and GraphPad 5.03. The statistical differences in the number of sperm cells with specific acrosomal status among control and experimental samples were assessed by the Kruskal–Wallis one-way analysis of variance (KW-ANOVA). Post hoc analysis was performed by the Newman-Keuls test and multiple comparisons of mean ranks. The Bland-Altman method was used to calculate the bias and its variance between the number of capacitated cells detected by individual methods after 240 min of incubation and the number of acrosome-reacted sperm after *ZP*-induced AR. The p value equal or lower to 0.05 was considered to be significant.

Results

Fluorescent microscopy detection of capacitation progress by individual methods

Figures 1 and 2 summarize data from fluorescent microscopy analysis of capacitation progress by presenting the percentage of cells with specific fluorescent pattern (% pattern) as detected by CTC, ACR.2, anti-pY (also Additional file 1: Figure S1) and FITC-phall (Fig. 1) at different incubation time (Fig. 2) from 20 individual samples ($n = 20$). At the beginning of the capacitation process (time 0 min), there was 5–8% of sperm with specific fluorescent pattern evaluated as capacitated and 7% of sperm evaluated as acrosome reacted and there were no significant differences between individual methods. At 120 min, a significant increase in the number of sperm with capacitated fluorescent pattern was observed in all the methods with the highest increase in ACR.2 and CTC. Moreover, at 240 min, all the methods detected significant increase in the number of capacitated sperm. After *ZP*-induced AR, all the methods detected strong significant decrease in the number of capacitated sperm, which correlated with the sperm evaluated for the specific fluorescent staining pattern after AR.

Flow cytometry detection of capacitation progress by individual methods

Figure 3 summarizes data from flow cytometry analysis of capacitation progress by presenting the flow cytometry histograms of the intensity in the FITC channel and the percentages of cells in appropriate gate at the beginning of capacitation (time 0 min), at the end of capacitation (time 240 min) and after the *ZP*-induced AR. Fluorescent intensity increased in all methods during

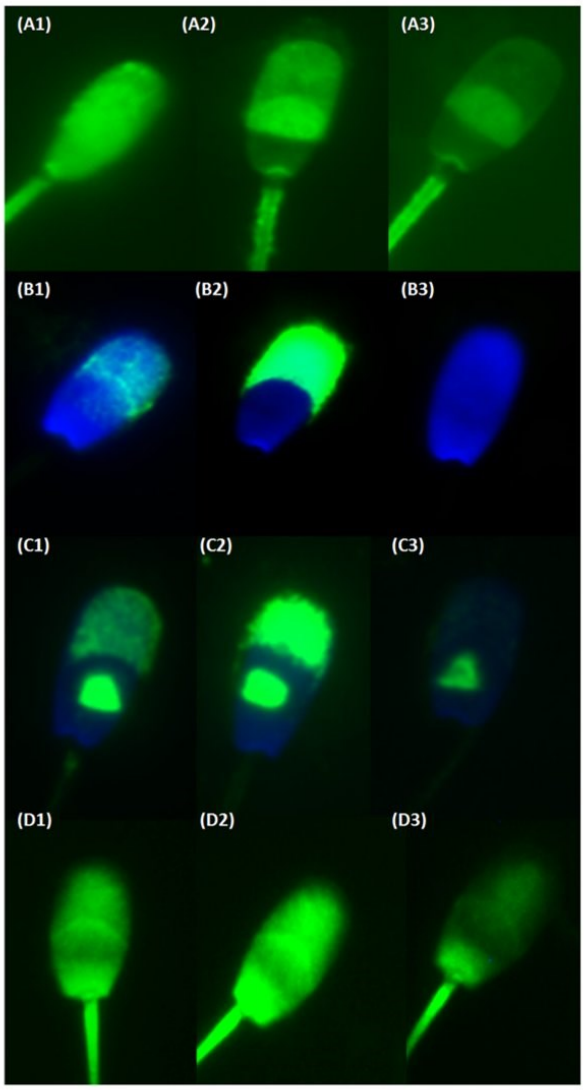


Fig. 1 (See legend on next page.)

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Fig. 1 Fluorescent microscopy pictures of sperm stained with CTC, ACR.2, anti-pY and FITC-phall. Acrosomal and sperm head fluorescent patterns prominent in distinct stages of capacitation process. a1 – a3 sperm treated by CTC: a1 Non-capacitated, acrosome-intact sperm - bright fluorescence over the entire sperm head and positive mid-piece of the tail; a2 Capacitated, acrosome-intact sperm - prominent fluorescent positive equatorial segment and mid-piece, fluorescence-free (dark) band in the post-acrosomal region; a3 Acrosome-reacted sperm - low fluorescent signal throughout the sperm head, with a remaining positive signal in the equatorial segment and mid-piece. B1 – B3 representative pictures of three specific ACR.2 acrosomal fluorescent patterns: b1 Non-capacitated, acrosome-intact sperm - moderate uniform fluorescence in the acrosomal area; b2 Capacitated, acrosome-intact sperm - intensive fluorescence of the acrosome; b3 Acrosome-reacted sperm - low or no fluorescent signal in the sperm head. Anti-pY: C1 – C3 pictures of three specific pY staining patterns: c1 Non-capacitated sperm – moderate signal in the acrosomal area, visible triangular segment; c2 Intensive fluorescence of the sperm head, triangular segment and tail – capacitated, acrosome-intact sperm; c3 Very low/no signal in the acrosomal area, visible triangular segment – acrosome reacted sperm. D1 – D3 representative pictures of three specific FITC-phall staining: d1 Non-capacitated sperm – moderate fluorescence in the acrosomal and sperm head/tail area; d2 Intensive fluorescence of the acrosome and the tail – capacitated, acrosome-intact sperm; d3 Low intensity in the acrosomal and apical sperm head area – sperm after AR. b1 – b3, c1 – c3 nuclei stained with a Blue DAPI dye

capacitation progress with the exception of the CTC assay, where fluorescent intensity was highly stochastic and was not subjected to subsequent gating and analysis (panel D was subsequently used for correlation analysis for CTC absolute fluorescent intensity). The ACR.2 detection method (Fig. 3a) displayed three completely separated peaks corresponding to the non-capacitated (NC), capacitated (C) and acrosome-reacted (AR) sperm populations and indicated the highest differences between the numbers of NC, C and AR sperm. On the other hand, pY detection method (Fig. 3b) provided three well distinguishable, but not completely separated peaks for NC, C and AR sperm populations. The overlap among individual intensity peaks also led to the smaller differences in the percentage of the individual sperm populations. The similar output was provided by the Phall detection method (Fig. 3c) with slightly higher overlap between individual intensity peaks especially for the NC/AR sperm populations.

Correlation of individual methods

Table 2 represents the correlation between a number of capacitated sperm cells among individual detection methods. All the methods expressed significant correlation ($p \leq 0.05$) with exception of flow cytometry for CTC. The highest correlation was observed between fluorescent microscopy detection of the capacitation state by CTC and ACR.2 antibody. Even though there was a high Pearson correlation coefficient ($r = 0.81$) for fluorescent microscopy data, the individual methods

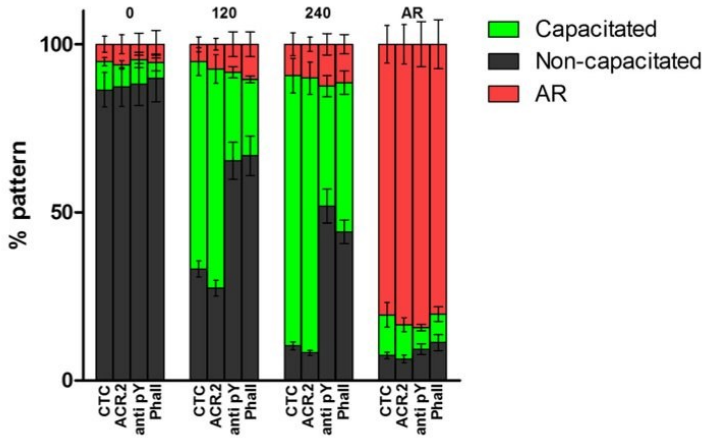


Fig. 2 Percentage of non-capacitated, capacitated and acrosome-reacted sperm analyzed by FC after different incubation time in capacitation medium (0, 120 and 240 min) and *ZP*-induced AR. Individual bars denote the percentage of non-capacitated, capacitated and AR cells as detected by individual methods among pre-defined sequential times of the capacitation and after AR. Samples from 20 individual boars were analyzed in this assay. No significant difference among individual methods at 0 min and after AR. Significant difference between CTC/ACR.2 vs anti-pY/FITC-phall at 120 and 240 min ($p \leq 0.05$). The percentage of capacitated sperm differed ($p \leq 0.05$) among end points (0, 120, 240, and AR) for the same evaluation method. Error bars indicate SEM

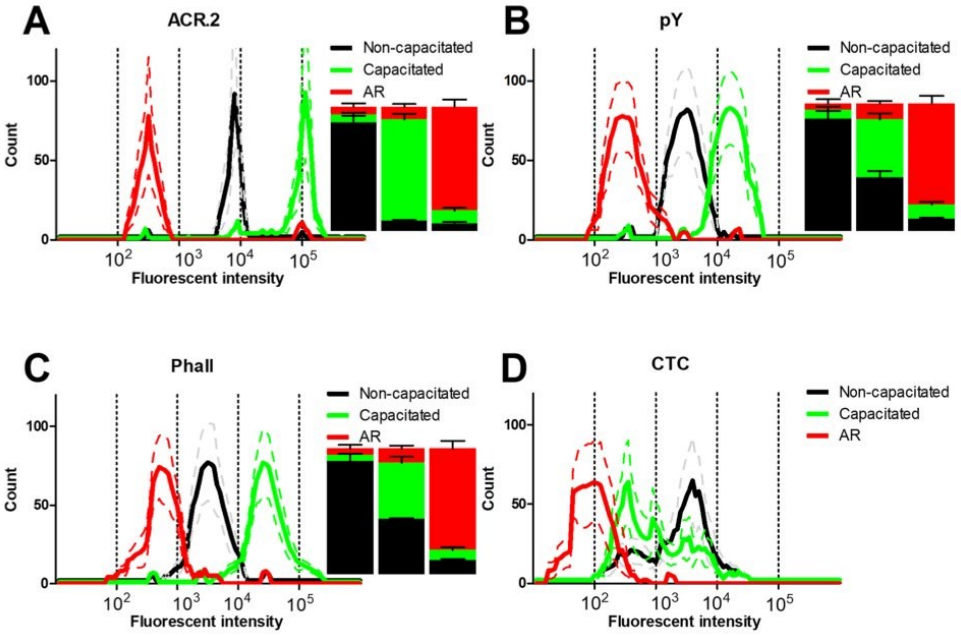


Fig. 3 Flow cytometry histograms and percentage of non-capacitated, capacitated and AR cells. Histograms and bar charts from the flow cytometry analysis of the non-capacitated (black), capacitated (240 min; green) and AR sperm (red) as analyzed by ACR.2 (**a**), anti-pY (**b**), FITC-phall (**c**) and CTC (**d**). The histograms represent the fluorescent signal intensities in 10.000 cells in non-capacitated, capacitated and AR among the analyzed samples ($N = 20$). Inserted bars denote the corresponding distribution of the percentage of the non-capacitated, capacitated and AR cells at time 0 (left), 240 min (middle) and after AR (right). Bar graphs are not show for the CTC assay

expressed different sum of correlation coefficients (r total) with the highest r total for CTC and ACR.2 fluorescent methods. In contrast, flow cytometry with CTC and fluorescent microscopy with FITC-phall had the lowest r total (r total = 2.51 and 3.34). In general, individual data sets from fluorescent microscopy expressed higher in-between correlation compared to coefficients between fluorescent microscopy (FM) and flow cytometry (FC) data and oppositely.

Correlation between number of capacitated cells detected by individual methods and number of cells after *ZP*-induced acrosome reaction (AR)

Table 3 represents the correlation between number of capacitated cells after 240 min of incubation and number of cells after *ZP*-induced acrosome reaction. The Pearson correlation coefficients (r) between number of cells with specific acrosomal pattern and means of fluorescent intensity (detected by CTC, ACR.2, anti-pY and FITC-phall) and numbers of cells after AR (detected by PSA fluorescent microscopy (PSA FM) and flow cytometry (PSA FC)) are presented. The strongest correlation was observed between a number of capacitated cells detected by CTC fluorescent microscopy (CTC FM) and a number of cells after AR detected by fluorescent microscopy with PSA (PSA FM). Almost the same result was obtained by fluorescent microscopy with ACR.2 antibody (ACR.2 FM) and PSA FM. All other methods and approaches expressed correlation at different levels of significance with exception of flow cytometry with CTC. In general, individual data sets from fluorescent microscopy expressed higher in-between correlation compared to coefficients between FM and FC data.

Figure 4 graphically summarizes the correlations between the percentages of capacitated sperm at 240 min of incubation detected by individual fluorescent microscopy methods and percentage of AR sperm after the *ZP*-induced AR detected by PSA method. The highest Pearson correlation coefficient was observed by CTC analysis (r = 0.93) and ACR.2 method (r = 0.92) while FITC-phall assay and pY assay presented moderate positive correlations (r = 0.68 and r = 0.5, respectively). All the correlation

Table 2 Correlation matrix of individual detection methods of boar sperm (chilled 17 °C/diluted) capacitation status at 240 min of incubation; $n = 20$

	ACR.2 FM	ACR.2 FC	CTC FM	CTC FC	Phall FM	Phall FC	pY FM	pY FC
ACR.2 FM[a]	**1**	**0.79**	**0.81**	0.19	**0.33**	**0.55**	**0.47**	**0.55**
ACR.2 FC[b]	**0.79**	**1**	**0.69**	0.17	**0.33**	**0.62**	**0.39**	**0.44**
CTC FM[c]	**0.81**	**0.69**	**1**	0.25	**0.35**	**0.59**	**0.52**	**0.47**
CTC FC[d]	0.19	0.17	0.25	**1**	0.24	0.31	0.12	0.23
Phall FM[e]	**0.33**	**0.33**	**0.35**	0.24	**1**	**0.47**	0.27	**0.35**
Phall FC[f]	**0.55**	**0.62**	**0.59**	0.31	**0.47**	**1**	0.23	0.29
anti-pY FM[g]	**0.47**	**0.39**	**0.52**	0.12	0.27	0.23	**1**	**0.71**
anti-pY FC[h]	**0.55**	**0.44**	**0.47**	0.23	**0.35**	0.29	**0.71**	**1**
r total[i]	**4.69**	**4.43**	**4.68**	**2.51**	**3.34**	**4.06**	**3.71**	**4.04**

Correlation coefficients (r) between detections of capacitation status by individual detection methods
[a]Fluorescent microscopy with anti-acrosin (ACR.2 FM) antibody
[b] Flow cytometry with ACR.2 antibody (ACR.2 FC)
[c]Fluorescent microscopy with chlortetracycline (CTC FM)
[d]Flow cytometry with chlortetracycline (CTC FC)
[e]Fluorescent microscopy with fluorescein isothiocyanate-conjugated phalloidin (Phall FM)
[f]Flow cytometry with fluorescein isothiocyanate-conjugated phalloidin (Phall FC)
[g]Fluorescent microscopy with anti-phosphotyrosine antibody (anti-pY FM)
[h]Flow cytometry with anti-phosphotyrosine antibody (anti-pY FC)
[i]The sum of correlation coefficients for appropriate detection method
Significant correlation coefficient in bold ($p \leq 0.05$)

Table 3 Correlation matrix between % of capacitated boar sperm (chilled 17 °C/diluted) after 240 min of incubation detected by individual methods and number of cells after *ZP*-induced acrosome reaction detected by PSA; $n = 20$

	PSA FM[i]	PSA FC[j]
ACR.2 FM[a]	**0.92**	**0.69**
ACR.2 FC[b]	**0.77**	**0.84**
CTC FM[c]	**0.93**	**0.72**
CTC FC[d]	0.19	0.21
Phall FM[e]	**0.68**	**0.35**
Phall FC[f]	**0.54**	**0.62**
anti-pY FM[g]	**0.5**	**0.60**
anti-pY FC[h]	**0.64**	**0.71**

Correlation coefficients (r) between detections of capacitation status by individual detection methods and detection of cells after *ZP*-induced acrosome reaction detected by appropriate methods
[a]Fluorescent microscopy (FM) with anti-acrosin (ACR.2) antibody
[b]Flow cytometry (FC) with ACR.2 antibody
[c]Fluorescent microscopy with chlortetracycline (CTC FM)
[d]Flow cytometry with chlortetracycline (CTC FC)
[e]Fluorescent microscopy with fluorescein isothiocyanate-conjugated phalloidin (Phall FM)
[f]Flow cytometry with fluorescein isothiocyanate-conjugated phalloidin (Phall FC)
[g] Fluorescent microscopy with anti-phosphotyrosine antibody (anti-pY FM)
[h]Flow cytometry with anti-phosphotyrosine antibody (anti-pY FC)
[i] Fluorescent microscopy with *Pisum sativum* (PSA FM)
[j]Flow cytometry with *Pisum sativum* (PSA FC)
Significant correlation coefficient in bold ($p \leq 0.05$)

coefficients were statistically significant ($p \leq 0.01$). Inserted bar charts (Fig. 4) represent the relative numbers of capacitated cells at 240 min of incubation detected by corresponding method (C240), the relative number of AR sperm detected by PSA assay after the *ZP*-induced AR (AR) and their differences (Δ).

Data from FM experiments are finally represented as a Bland-Altman plot (Fig. 5; Additional file 3: Figure S3) which shows the agreement among individual methods. The zero baseline represents the percentage of cells detected as AR by PSA. All the methods underestimated the number of acrosome-reacted sperm and there were major differences in the calculated bias for individual methods. The lowest bias between the number of cells detected as capacitated after 240 min of incubation and number of acrosome-reacted cells after *ZP*-induced AR was calculated for ACR.2 and CTC method (5.2 ± 1 and 5.35 ± 0.87), the highest bias was calculated for pY method (46.78 ± 2.15).

Discussion

Spermatozoa have to undergo series of controlled molecular changes in female reproductive tract or in vitro before being able to bind, penetrate and fertilize the egg [1–3]. Nevertheless, many molecular and physiological aspects of capacitation are still waiting to be discovered or characterized. In our study, we targeted depiction of capacitation process dynamics by multiple fluorescent techniques and compare their detection outcome. Moreover, we were able to address the ability of individual

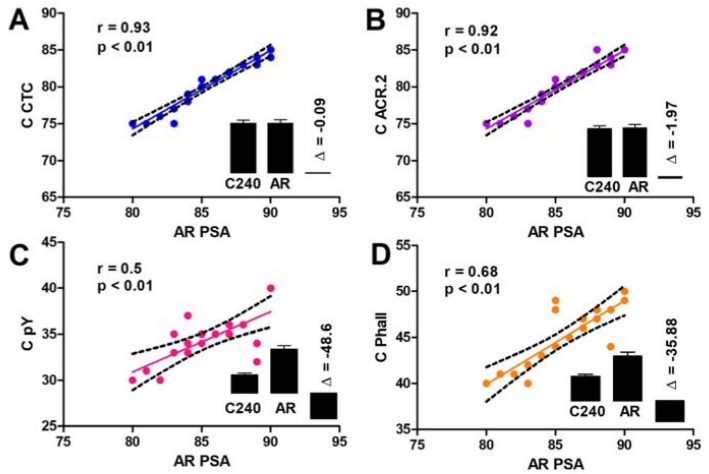


Fig. 4 Correlations and differences between the % of capacitated boar sperm at 240 min detected by FM by individual methods and % of AR cells detected by PSA FM. Individual graphs show the correlation lines, the correlation coefficient r and its *p*-value for the CTC (**a**), ACR.2 (**b**), pY (**c**) and FITC-phall (**d**). The inserted bars represent the comparison of the percentage of cells detected as capacitated by individual methods (C240), the percentage of the cells detected as AR by PSA assay (AR) and their difference (**Δ**). 20 chilled (17 °C) / diluted boar sperm samples were analyzed for each assay (*N* = 20)

methods to detect the measurable physiological status of capacitated sperm.

CTC is considered as gold standard in fluorescent microscopy analysis of sperm capacitation state [12, 13, 21–23]. Notable disadvantage of this method is the difficult assessment of individual cell fluorescent patterns under the fluorescent microscope [12] and a relative low fluorescent intensity in combination with fast photobleaching, which make analysis difficult for human eye. On the other hand, ACR.2 antibody analysis of the specimen is much easier for the human evaluator, due to a strong positive signal and prominent acrosomal patterns. Although anti-pY and FITC-phall are able to detect changes during capacitation in fluorescent intensity in the sperm head and tail, the major disadvantage of these methods is the absence of a specific fluorescent pattern corresponding to

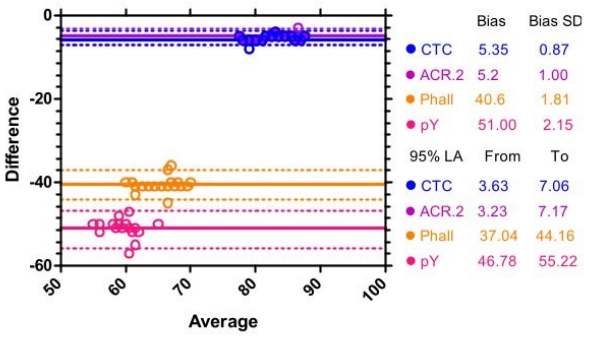


Fig. 5 Bland-Altman plot. Bland-Altman plot shows the differential bias between the percentage of cells detected as capacitated by individual methods after 240 min of incubation and the percentage of cells detected as acrosome-reacted by PSA after *ZP*-induced AR. The zero baseline represents the percentage of cells detected as acrosome-reacted by PSA, individual color lines shows the biases for CTC, ACR.2, FITC-phall and pY assays. Circles represents individual data points (*N* = 20 for each method), dotted lines represent 95% LA (Limits of agreement)

the capacitation progress and subsequent necessity to set an intensity threshold, which is subjective. However, this disadvantage may be overcome by using a computer picture analyzer [24]. The described challenges using anti-pY and FITC-phall methods for detection of the capacitation state resulted in lowest correlation of the data compares to those obtained by CTC and ACR.2.

The other obvious way how to overcome subjective analysis of the fluorescent intensities is to use flow cytometry. On very positive terms, in general, the flow cytometry data corresponded with those from fluorescent microscopy, with few important remarks. CTC assay may not be suitable for fluorescence detection by flow cytometry. During capacitation, the prominent change in the CTC fluorescent analysis is the appearance of the dark postacrosomal segment, a fluorescent pattern, which is not well distinguishable by cytometer detector. On the other hand, data from anti-pY and FITC-phall express much better statistical differences among individual capacitation times using flow cytometry, which might be likely due to the fact, that strong point of the flow cytometry analysis is the ability to precisely measure small differences in the fluorescent intensity. Finally, the strength of analysis using ACR.2 antibody is in the regular presence of three easily distinguishable fluorescent intensity peaks, which enables to gate them to acquire another set of useful data for statistical analysis. In general, flow cytometry generates different type of statistical parameters (e.g. arithmetical, geometrical mean of fluorescent intensity, number of accidents in set gates etc.), which are accessible for subsequent statistical analysis (e.g., comparing multiple groups by ANOVA) [17, 23, 25–27]. In our study, we analyzed the percentages of sperm in the appropriate gate for ACR.2, pY and Phall and arithmetical means of fluorescent intensity for CTC. In general, the strong point of flow cytometry analysis is ability to analyze thousands of cells per sample, objective analysis and capability to precisely measure the fluorescent intensity, which changes correlate with the physiological process. The relative weaknesses of the method are the cost of the instrument and analysis and inability to exactly asses the specific morphological fluorescent patterns, a drawback, which can be now almost overcome by sophisticated cytometers, which combine the advantages of both flow cytometers and fluorescent microscopes [28, 29].

The combination of the data from fluorescent microscopy and flow cytometry enables us to describe the temporal changes and succession of the molecular processes detected by individual analytical methods. According to our results, the first observable change is the redistribution of calcium ions (CTC FM [30];), accompanied by highest accessibility of acrosin epitopes (ACR.2 FM, FC), which resulted from enzymatic and proteomic changes in acrosomal matrix. At later capacitation stages (180 min), the phosphorylation of sperm proteins [31] and actin polymerization [6, 7] are also well detectable by presented methods. At this point it is important to mention, that collecting samples at only five different times during capacitation is not sufficient for detailed characterization of the molecular changes, on which physiological process of capacitation is based and sperm life imaging is more appropriate method to study this in detail. For example, fast changes in calcium concentration should be measured by methods other than CTC [32, 33]. Similarly, changes in actin polymerization should be measured by multiple analytical methods, since staining with FITC-phall can reflect rather changes in accessibility of actin epitopes than actin polymerization and depolymerization itself. On the other hand, CTC, contrary to methods measuring fast changes in calcium concentration, is able to reflect global changes of sperm cellular calcium homeostasis thus, likewise other methods used in this work, play important role in studying capacitation as cellular physiological process.

Due to the fact that capacitation is the physiological process, which results in the ability of sperm to undergo AR in the presence of *zona pellucida*, we tested the ability of individual methods to predict number of physiologically capacitated sperm. According to the results presented in Fig. 4 and Table 2, all used methods with well-thought of experimental design (fluorescent microscopy and flow cytometry) show a good correlation with the number of cells after *zona pellucida* induced AR, but there are major differences in their ability to predict the percentage of cells undergoing acrosome reaction in the presence of *zona pellucida* in boars. FM CTC and FM ACR.2 are best in prediction of status physiologically capacitated sperm showing the lowest bias in Bland-Altman analysis and thus can be used as a useful tool for optimization of capacitating media [34] or for studying effect of various compounds with the pro-, or anti-capacitation effect [14]. On the other hand, pY method showed the lowest agreement (the highest bias) between the number of cells detected as capacitated at 240 min and the number of cells detected as AR after *ZP*-induced acrosomal reaction and therefore in our arrangement highly underestimate the % of cells which will undergo the *ZP*-induced AR.

Despite the fact, that our experimental approach enabled to compare four methods used for characterization of capacitation process in boar sperm, and broaden up the knowledge on interpretation of obtained data, there are still several limitations, which need to be addressed in future studies. The first is related to the evaluation of individual cells in a sample by multiple analytical methods. The co-staining of the individual samples by for example ACR.2 and anti-pY antibody would enable to conclude if individual cells are detected by both

methods as non-capacitated, capacitated or AR and rigorously calculate methods agreement on the level of individual cells. This approach would not be technically possible for the CTC method since sample processing and evaluation by FM differs from antibody or FITC-phall staining. The second limitation is similar but related to AR prediction. The experimental approach used in the current study also does not allow to determine if individual cells detected as capacitated by individual methods would be exactly those undergoing AR when exposed to solubilized *ZP*. The presented ratios and agreements of cells detected as capacitated by CTC and ACR.2 and cells detected as AR by PSA after *ZP*-induced AR suggest that cells detected as capacitated by these two methods will undergo AR after exposure to solubilized *ZP*. However, such conclusion can not be drawn for anti-pY and FITC-Phall methods. A possible approach to probe this in further detail would be to induced AR by *ZP* during several times of incubation where the ratios of cells detected as non-capacitated and capacitated are different and using FC observed what population of cells (non-capacitated/capacitated) will undergo AR. However, there are again several technical limitations, since ACR.2 antibody displays intermediate fluorescent intensity peaks in earlier stages of the incubation and there are gates overlaps for anti-pY and FITC-phall, as shown in Fig. 3. Similarly, the presented approach would not suit the CTC method.

To summarize, the multiple fluorescent methods used in our study to monitor boar sperm capacitation proved to be able to detect the temporal changes of the capacitation process. However, for some methods, flow cytometry is more appropriate than fluorescent microscopy and vice versa, and this should be considered in an experimental design. Data from individual analytical methods significantly correlates, albeit there are notable differences in the correlation coefficient between them. Furthermore, a change in the temporal dynamics in individual molecular processes detected by appropriate methods were observed. These individual observations and assessments are crucial, as the differences in temporal changes allow us to make rough model of chronological succession of processes underlying capacitation. Finally, using a correlation analysis with data from *ZP*-induced acrosome reaction was shown, that described methods are able to predict the number of spermatozoa undergoing AR after exposure to *ZP* but there were major differences among individual methods. The detailed knowledge of limits of these methods commonly used for evaluation of capacitation status and prediction of sperm ability to undergo the AR should help to standardize individual results and lead to production of good comparable data among scientific laboratories.

Conclusions

Capacitation is one of the most crucial steps sperm has to undergo before being able to fertilize egg. Therefore, the proper characterization of its dynamics has great importance for many studies addressing sperm physiology. In this article, we have studied capacitation of boar sperm using four largely used methods, compared their experimental outputs using fluorescent microscopy and flow cytometry and highlighted their limits and differences when detecting capacitation progress. Furthermore, we show that CTC and ACR.2 methods are able to accurately predict the percentage of acrosome-reacted sperm after *ZP*-induced AR. Our study thus contributes further to better characterization of the important step in the mammalian reproduction such as capacitation.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12958-019-0554-z.

Additional file 1: Figure S1. Example of the fluorescent microscopy analysis of the sperm capacitation status. The microphotograph shows 14 boar sperm stained by anti-pY antibody (green) and counterstained by DAPI. Eleven cells detected as capacitated (C), two cells detected as non-capacitated (NC), one cell as AR. Time 240 min, 400x.

Additional file 2: Figure S2. Example of the flow cytometry analysis of the boar sperm capacitation progress by ACR.2 antibody. The dot plots represent the gating of the analyzed sperm population using FSC and SSC detection and the histograms of the FITC channel with the gating of the non-capacitated (N), capacitated (C) and AR cells (%) for the individual time points (0, 30, 60, 90, 120, 180, 240) and AR. The data from 0, 240 and AR from the 20 individual boars were subsequently used for the preparation of Fig. 3.

Additional file 3: Figure S3. Bland-Altman plots separately for individual methods. Bland-Altman plots (decomposed Fig. 5) show the absolute bias between the percentage of cells detected as capacitated by individual methods after 240 min of incubation and the percentage of cells detected as acrosome-reacted by PSA after *ZP*-induced AR. The zero baseline represents the percentage of cells detected as acrosome-reacted by PSA, solid lines shows the absolute biases for CTC, ACR.2, FITC-phall and pY assays. Dots represent individual data points, dotted lines represent 95% LA (Limits of Agreement).

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Authors' contributions
LD conceptualization, supervision, investigation, data processing, writing manuscript; PD investigation, methodology, data acquisition, writing manuscript; LD and PD contributed to the project equally. EZ investigation, methodology, data curation, writing manuscript; AD investigation, methodology, data acquisition; KK conceptualization, supervision, funding acquisition, writing manuscript; JP conceptualization, supervision, funding acquisition, investigation, writing manuscript. All authors read and approved the final manuscript.

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The data and materials are available from the corresponding author on reasonable requests.

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interest.

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Supplement 3

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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.

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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

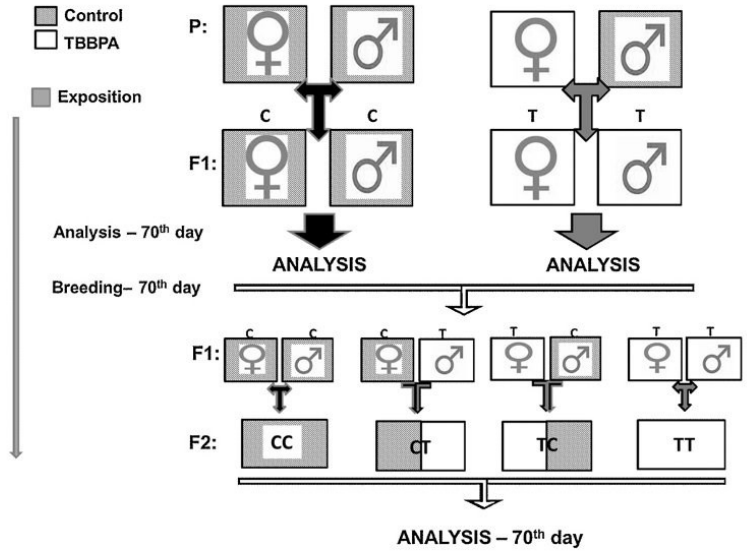


Fig. 1. Breeding diagram. Animals in parental generation were bred to form the F1 generation consisting of two groups (**C**; **T**). In group **C**, born pups were not affected by TBBPA. In group **T**, the pups were exposed to TBBPA in utero and postnatally. Animals of the F1 generation were bred to 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

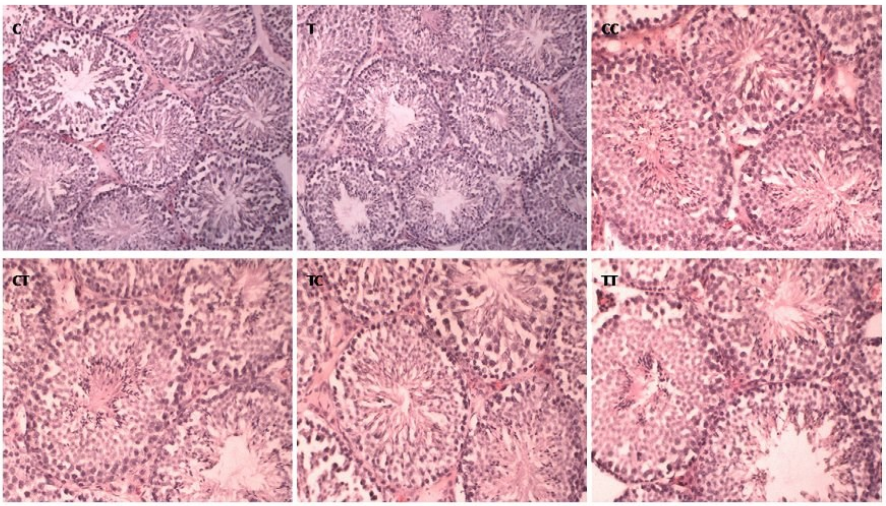


Fig. 2. Histological analysis of the testes from control and experimental animals. Paraffin sections were placed onto glass slides and stained by eosin (cytoplasm) and haematoxylin Harris (nucleus). Histological samples were analyzed under the microscope. Normal morphology and the process of spermatogenesis 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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Supplement 4

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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.

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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	TTGCAGCTTGTCGGGGACAGCA 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	CCACTGCCGAGGAACGGAAGC CGTTGCCGGAACCTCGGGACG	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).

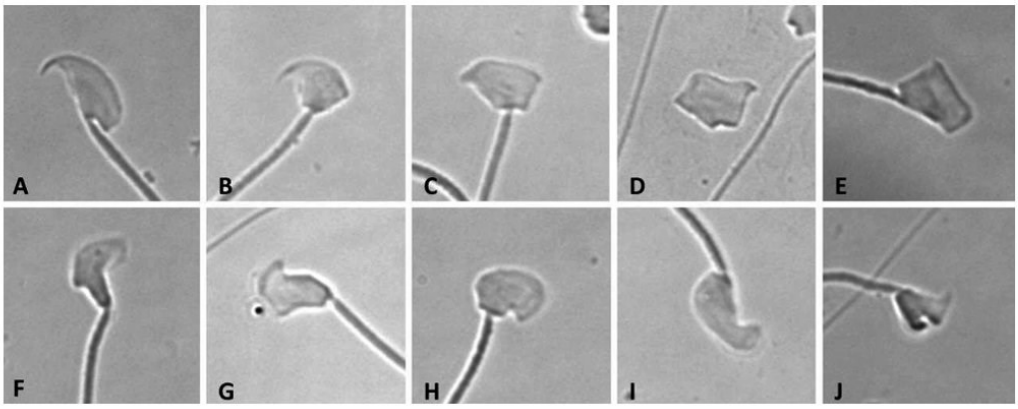


Fig. 1. Exemplary pictures from light microscopy showing sperm with normal (A) and aberrant morphology (B–J).

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.

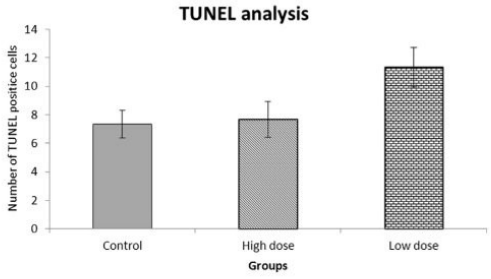


Fig. 2. Number of TUNEL-positive cells in testicular tissue sections. The high-dose group was exposed to 150 μg/l of ZE; the low-dose group was exposed to 0.15 μg/l of ZE. Mean ± SD; $n = 18$.

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.

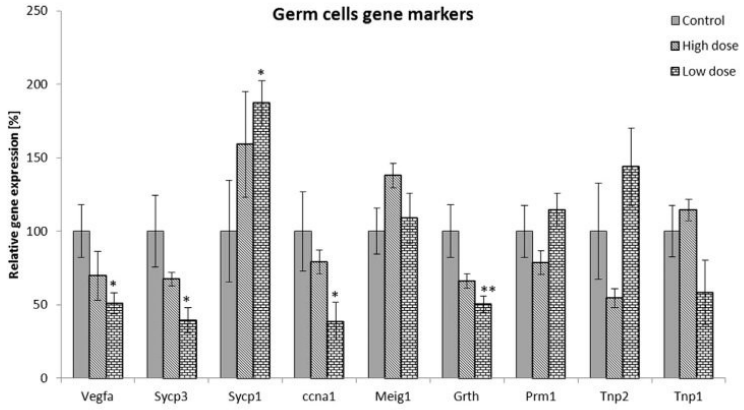


Fig. 3. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes expressed in testicular germ cells. In this study, genes for vascular endothelial growth factor (*Vegfa*), synaptonemal complex protein 3 (*Sycp3*), synaptonemal complex protein 1 (*Sycp1*), cyclin A1 (*Ccna1*), 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.

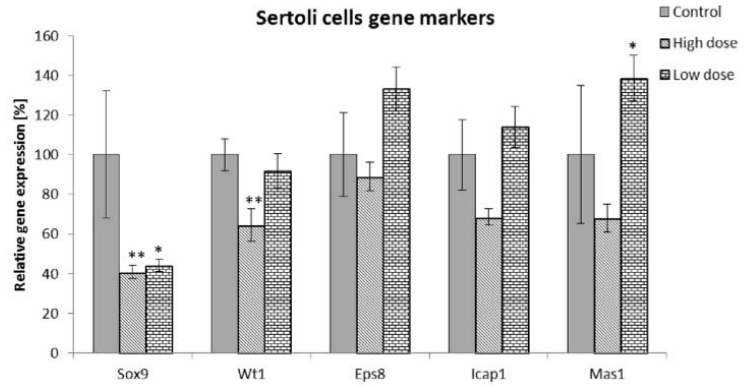


Fig. 4. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes expressed in Sertoli cells. In this study, genes for SRY-box containing gene 9 (*Sox9*), Wilms tumour 1 (*Wt1*), epidermal growth factor receptor pathway substrate 8 (*Eps8*), integrin beta 1 binding protein 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; $n = 9$.

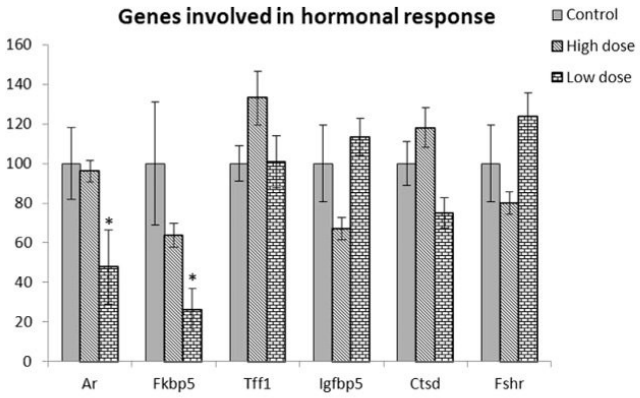


Fig. 5. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes playing a role in the response to various hormonal stimuli. In this study, genes for androgen receptor (*Ar*), FK506 binding protein 5 (*Fkbp5*), trefoil factor 1 (*Tff1*), insulin-like growth factor binding 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

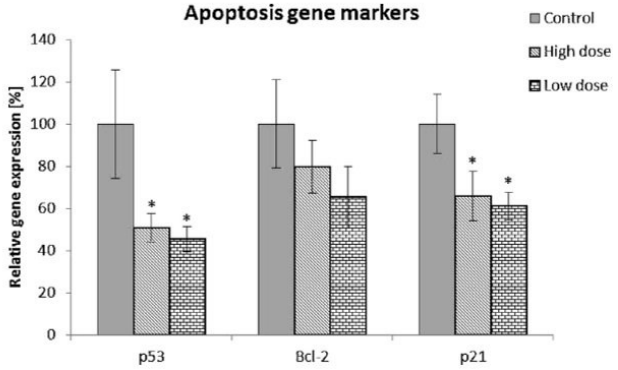


Fig. 6. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes playing a role in apoptosis. In this study, genes for tumour protein p53 (*p53*), B-cell leukaemia/lymphoma 2 (*Bcl2*) and cyclin-dependent kinase inhibitor 1A (*p21*) were tested. The graph illustrates the 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; $n = 9$.

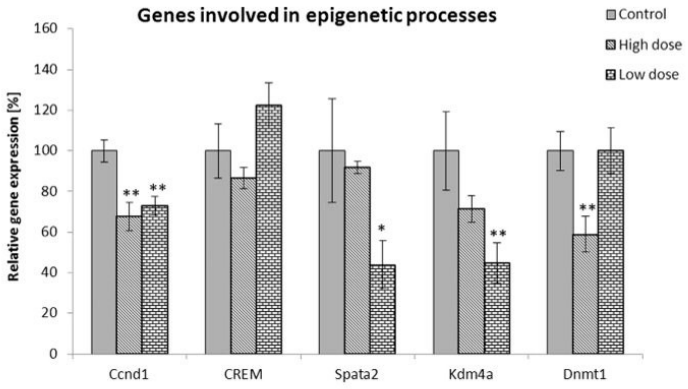


Fig. 7. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes that are related to epigenetic processes. In this study, genes for cyclin D1 (*Ccnd1*), cAMP responsive element modulator (*Crem*), lysine (K)-specific demethylase 4A (*Kdm4a*), spermatogenesis associated 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.

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Supplement 5

Dostalova P, Zatecka E, Ded L, Elzeinova F, Valaskova E, Kubatova A, Korenkova V, Langerova L, Komrskova K, Peknicova J. **Gestational and pubertal exposure to low dose of di-(2-ethylhexyl) phthalate impairs sperm quality in adult mice.** Reprod Toxicol. 2020 Sep;96:175-184. doi: 10.1016/j.reprotox.2020.06.014. Epub 2020 Jun 30. PMID: 32619501.

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Gestational and pubertal exposure to low dose of di-(2-ethylhexyl) phthalate impairs sperm quality in adult mice

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ABSTRACT

Di-(2-ethylhexyl)-phthalate (DEHP) is a compound widely used as a plasticizer, which can leach from plastics into the environment and thus influence human health. The aim of this study was to analyze whether exposure to an environmentally relevant dose of DEHP during mice fetal development or puberty can cause long-lasting changes detectable month/s after the last exposure. We used a DEHP concentration relevant to a daily human intake of 2.4–3 μg/kg of body weight/day. CD1 outbred mice were treated either *in utero* or postnatally during puberty and analyzed in adulthood. Analyzing fertility parameters using morphometric, histologic, genomic and proteomic methods we showed that DEHP exposure leads to decreased sperm concentration and quality, in both experimental groups. Moreover, the changes in anogenital distance, seminal vesicle weight, and testicular gene expression suggest a disturbance of androgen signaling in exposed animals. In conclusion, we hereby present, that the prenatal and pubertal exposure to a low dose of DEHP negatively influenced reproductive endpoints in male mice, and some of the effects were persistent until adulthood.

1. Introduction

Plastics are materials widely used in all aspects of modern life and may pose certain dangers to health. The risk could stem from the components of plastics such as phthalate plasticizers, which are additives that increase the flexibility and transparency of the material [1]. One of the phthalates considered dangerous to human health is DEHP. Since DEHP is not covalently bound to the polymer, it can leach from plastics and be available for biological exposure; for instance, up to 15 % of incorporated DEHP is reported to originate in medical devices [2,3]. Sources of exposure have also been found in food that had been in contact with food packaging, child products, and various industrial products [4–6]. Upon oral exposure, DEHP is rapidly metabolized by intestinal lipases and liver esterases to its monoester (MEHP), which can be further biotransformed to oxidative metabolites, glucuronidated, and excreted in the urine [7,8]. MEHP is ten times more potent than DEHP and responsible for much of DEHP´s toxicity [9]. However, not all DEHP and its metabolites are excreted, they can cross the placenta and have a direct effect on the developing embryo. DEHP has been detected in rat placenta, foetal tissue and amniotic fluid [10], and has also been detected in humans; specifically in umbilical cord blood [11,12], placenta [13,12], breast milk [14], amniotic fluid [8], and it is more frequently detected in fetal than in maternal serum [12]. It is important to note that the metabolic pathway of DEHP in humans is different from that of mice, especially the MEHP oxidation pathways [15]. However, the inter-individual variation in DEHP metabolism in humans may be greater than the difference between mice and humans [15].

It is assumed that DEHP affects hormonal balance, leading to developmental alterations. There are two critical testicular developmental periods during the ontogeny which are more sensitive to endocrine disruptors such as DEHP. The first is during the fetal development when the gonads are formed and sex is determined during the masculinization programming window (MPW), and when testosterone production by fetal Leydig cells begins [16]. After birth, testosterone production ceases and starts again with the onset of puberty, which represents the

Abbreviations: AGD, anogenital distance; BTB, blood-testis barrier; ES, ectoplasmic specialization; FSH, follicle stimulating hormone; DEHP, Di(2-ethylhexyl) phthalate; LH, luteinizing hormone; MEHP, mono(2-ethylhexyl) phthalate; MPW, masculinization programming window; NOAEL, no observed adverse effect level; SC, Sertoli cell; TBC, tubulobulbar complex

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second critical testis developmental period. Hormone imbalance during these periods leads to reproductive disorders.

Based on the fertility studies in mice, DEHP is the most potent reproductive toxicant among the phthalates [17]. The most commonly observed phenotypes after DEHP exposure were reduced anogenital distance and weight of reproductive organs, undescended testes, reduction in daily sperm production, testicular as well as epididymal malformations, changes in steroid hormone levels and sexual inactivity ([18–23]; C [3,24–26].). The effect of DEHP on mouse spermatogenesis may vary in a strain-dependent manner; FVB/N mouse strain is less sensitive than C57BL/6 J mice [23]. *In utero* exposure to DEHP also causes embryo toxicity and teratogenic effects in mice and rats ([27,28]). The adverse effect of DEHP on mouse reproduction is transgenerational as has been shown in studies in both sexes [29]. There is also evidence of the negative effect of DEHP on human reproduction [30]. The calculated median daily DEHP intake for human is approximately 1–4.2 μg/kg b.w. [17], which is below the no-observed-adverse-effect level (NOAEL) for DEHP that was determined at 4.8 mg/kg/day based on the monitoring of reproductive tract malformations in animals [31].

The aim of this study was to analyze whether DEHP exposure during two critical male developmental periods: masculinization programming window (MPW) and puberty, can cause long-lasting changes detectable month/s after the last exposure. Unlike previous studies, we used a concentration relevant to the human daily intake of 2.4–3 μg/kg b.w./day. We believe that our study will contribute to broader understanding of mechanism how DEHP affects the germ cell development and subsequently semen quality as well as whether the DEHP exposure leads to increased infertility in humans.

2. Materials and methods

2.1. Animals and treatment

CD1 (ICR) outbred mice (AnLab Ltd.) with higher heterozygosity were used for the experiments. The weaned males were randomly divided into groups of four animals (siblings housed together) and housed under standard experimental conditions (constant temperature (23 − 24 °C), humidity (60 ± 5%) and 12-h light regime) in the animal facility of the Czech Academy of Sciences, Institute of Molecular Genetics, Prague. Mice were fed on soya-free food without phytoestrogens (Ssniff, Soest, Germany). The diet and water were administered *ad libitum*. DEHP (Sigma cat. no. 36,735) was administered to drinking water (in concentration 20 μg/L) to attenuate all forms of perceived stress. DEHP was solved in 70 % ethanol and then it was diluted 1000x in drinking water. The controls were administered 1000x diluted 70 % ethanol in drinking water. Since we used a low DEHP concentration it was not necessary to use any other solvent. Reported daily water intake of an adult mouse is in rage between 4 mL [32,33] and 5 mL per day [34], therefore the calculated daily intake of DEHP in our study is between 2.4 and 3 ug/kg of bodyweight. Five female mice per group (3 months old) were bred and exposed orally to the DEHP during gestational days (GD 10–20). These animals formed group G. Only male descendants were included into this study. Another group of experimental animals (only males) were exposed to DEHP only postnatally (PD 21–45) and formed group P. Male mice without gestational or postnatal treatment were used as a control group (C). Twenty two male mice were evaluated in the control group and eighteen male mice were evaluated in groups exposed to DEHP. In animals treated *in utero* we used the litter as the statistical unit to remove potential litter effects. Subsequently, for gene expression and protein analysis we have selected one animal from each litter as a representative sample. This study was conducted in accordance with the Guide for the Care and use of Laboratory Animals (NIH Publication No.86 − 23, revised 1996). The experimental protocol was approved by the Animal Care and Use Committee of the Institute of Molecular Genetics, CAS and carried out in accordance with the regular relevant guidelines and regulations.

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.

2.3. Preparation of cells

Mouse sperm cells were obtained from the left and right cauda epididymis. Spermatozoa from both caudae were left to release into warm (37 °C) PBS in a CO_2 incubator for 15 min. The resulting mixture was poured through a 30 μm filter (Partec) to obtain only the sperm fraction and PBS was added to 1 mL of final volume. Sperm were counted in a Burker chamber under the light microscope [35]. Sperm motility was monitored under the light microscope. 20 μL of the spermatozoa was taken for assessing viability and morphology, and 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.

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 the Spermac Stain System (Ferti Pro). An indirect immunofluorescence using monoclonal antibodies (Hs-8 and Hs-14) against intra-acrosomal proteins in combination with nuclei staining was used to asses a morphology of the sperm head and acrosome under the fluorescence microscope [36,37]. To determine the viability of epididymal spermatozoa, a Live/Dead sperm viability kit (Invitrogen) was used according to the manufacturer's instructions with minor modifications that were previously described [38]. Samples were evaluated under a Nikon Eclipse E400 fluorescence microscope using a 40 x Nikon Plan Fluor 40/0.75. A minimum of 200 cells per animal were evaluated.

2.5. Indirect immunofluorescence

Monoclonal antibodies (MoAbs) against intra-acrosomal sperm proteins Hs-14 and Hs-8 prepared in our laboratory [36,37] were used to determine the acrosomal status of epididymal spermatozoa. Hs-8 antibody detects glyceraldehyde-3-phosphate dehydrogenase (GAPDHS), which is important for binding to the egg [39], and Hs-14 antibody detects transitional endoplasmic reticulum ATPase (TERA) [36,40]. Epididymal spermatozoa were smeared onto glass slides, air dried and fixed for 10 min with acetone. Slides were rinsed with PBS and incubated overnight with Hs-8 or Hs-14 MoAbs (diluted in 1% BSA in PBS to an immunoglobulin concentration of 20 μg/mL) at 4 °C. After thorough washing with PBS, smears were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM immunoglobulins (μ-chain specific; Sigma), 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). An Annexin V–FITC Apoptosis Detection Kit (Sigma) was used to label apoptotic sperm according to the laboratory instructions [38]. Samples were examined with a Nikon Eclipse E400 fluorescence microscope equipped with a Nikon Plan Apo VC 60/1.40 oil objective (Nikon Corporation Instruments Company) and photographed with a CCD 1300-VDS camera (Vosskühler) with the aid of the NIS-ELEMENTS Ar imaging software (Laboratory Imaging, Ltd.). A minimum of 200 cells

per animal were evaluated.

2.6. Histological analysis and TUNEL assay

The right testis of all animals was fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin-eosin. The tissue morphology and status of spermatogenesis were evaluated under the light microscope.

The numbers of apoptotic cells in the testis paraffin sections were detected by the terminal deoxynucleotidyl transferase-mediated dUTPnick end labelling (TUNEL) method using an *in situ* detection kit (Promega) according to the manufacturer's instruction. DAPI was used to visualize the nuclei. Samples were evaluated under a fluorescence microscope Nikon Eclipse E400, Nikon 40× Plan 40/0.65. The number of TUNEL-positive cells in 20 cross-sectioned seminiferous tubules was counted in all samples. Differences between the number of TUNEL-positive cells in the control and experimental samples were statistically evaluated.

2.7. Isolation of testicular RNA

The left testis was removed from the sacrificed animal, immediately placed into 1 mL of Tri-Reagent (Sigma) and homogenized in homogenizer Precellys 24 (Bertin Technologies) for 3 × 20 s, 5000 rpm. Subsequently, total RNA was isolated by a Tri-Reagent kit according to the manufacturer's instructions (Sigma). Isolated RNA was dissolved in nuclease-free water (Fermentas) and stored at −70 °C. The RNA quality and purity was evaluated in a spectrophotometer (Helios α, Thermo Electron Corporation). The integrity of isolated RNA was analyzed in Fragment Analyzer (Advanced Analytical).

2.8. Reverse transcription (cDNA synthesis)

For cDNA synthesis, 2 μg of isolated RNA were added to 1 μl of buffer DNase I + $MgCl_2$ (Fermentas), 1 μl DNase I (Fermentas) and H_2O to reach a volume of 10 μl. This mixture was incubated for 30 min at 37 °C. Subsequently, 1 μl of EDTA was added and the mixture was incubated for 10 min at 65 °C. Next, 4 μl of reaction buffer for M-MuLV reverse transcriptase (Fermentas); 2 μl of 10 mM dNTPs (Fermentas); 2 μl Universal RNA Spike I template (TATAA Biocenter); 1 μl oligo(dT) + random hexamer primers (Fermentas); 0.5 μl Ribolock inhibitor (Fermentas), and 1 μl of M-MuLV reverse transcriptase (Fermentas) were added and this mixture was incubated for 60 min at 42 °C followed by 10 min at 70 °C in a thermocycler (Techne Touchgene Gradient PCR Thermal Cycler, Labrecyclers). After the incubation, the mixture was cooled down to 4 °C and kept in −20 °C until next analysis. A control without reverse transcriptase (no RT control) was prepared for each sample to exclude possible contamination with genomic DNA.

2.9. BioMark and real-time qPCR

The BioMark high-throughput microfluidic qPCR platform (Fluidigm) was used to analyse the gene expression as previously described [38]. The used primers are summarized in Table S1. To complement the mRNA study, real-time qPCR analysis of genes related to the blood-testis barrier (BTB), ectoplasmic specialization (ES), and tubulobulbar complex (TBC) was performed using a CFX 384-qPCR cycler (BioRad). The used primers are summarized in Table S2. Each reaction mix contained 2 μL cDNA (20 ng RNA/μl), 5 μL SYBR Green Master Mix (Fermentas), 1 μL primers, and 2 μL H_2O. The thermal qPCR protocol was: initial denaturation at 95 °C for 10 min.; 35 cycles of 95 °C for 15 s, 60 °C for 30 s, and 75 °C for 30 s. All reactions were performed in duplicates. All primers were tested for their efficiency, and only primers with efficiency close to 100 % were used for further analysis. The spike control and no RT control were run for each sample to analyze the possible inhibition and genomic DNA contamination, respectively. No template control was run for each primer to analyze primer-dimer formation and possible contamination. The β-actin gene (*Actb*) was selected by Normfinder (GenEx Enterprise, MultiD Analyses) and used as the reference gene. The data were analyzed with GenEx Enterprise, MultiD Analyses. The relative expression of a target gene was calculated based on the quantification cycle (Cq). First, data were normalized to the reference gene, then they were converted into relative quantities expressed relative to the sample with the lowest expression (maximum Cq), and finally converted to a logarithmic scale with base 2.

2.10. Protein isolation and western blot

Testicular proteins were isolated from Tri-Reagent-treated samples (Sigma). First, the proteins were precipitated in ice-cold acetone, dissolved in 10 % SDS, and further processed according to the manufacturer's instructions. The final pellet was dissolved in Laemmli sample buffer. Equal amounts of proteins were loaded onto 4–20 % gradient gel (BioRad), run, and subsequently transferred to PVDF membrane (Immobilon P, Millipore). Five % milk in Tween-PBS was used as a blocking agent. Immunodetection was performed with the following antibodies: goat anti-3β-HSD (P-18), sc-30,820 (SantaCruz), (1:200); mouse anti-α tubulin, clone DM1A, T9026 (Sigma), (1:5000); goat anti mouse-HRP (BioRad), (1:3000); rabbit anti goat-HRP (BioRad), (1:3000). Incubation with primary antibodies was performed overnight at 4 °C, with secondary antibodies at room temperature for 1 h. Protein expression was visualized by SuperSignal (ThermoScientific) and detected in Azure c600. Densitometry was performed using ImageJ [41]. Signal intensity of the examined protein was normalized to the signal intensity of the loading control protein (α-tubulin). The control was set at 1 and the experimental samples were compared to the control. Six samples were evaluated per group.

2.11. Statistical analysis

The statistical differences among the compared groups were analyzed using STATISTICA 7.0. (Statsoft, Czech Republic). When using parametrical tests, the normal distribution of the data was tested. Differences between AGD, body weight, and sperm concentration were analyzed 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 the number of TUNEL-positive cells were assessed by KW ANOVA and multiple comparisons of mean ranks. The differences in the relative expression of tested genes were analyzed by one-way ANOVA and Dunnet post-hoc test. In P group, individual animals were used as statistical unit (n) and in the G group the statistical unit was the dam averages (n); individual values were then compared to control group C accordingly (P - individual C animals / G—C dam averages). The P value < 0.05* (< 0.01**, < 0.001***, respectively) was considered as significant.

3. Results

3.1. Reproductive parameters

DEHP treatment resulted in a shorter anogenital distance in both gestation (G) and pubertal (P) groups and a reduced weight of seminal vesicles in group P. Further, we did not observe any changes in the number of progeny, sex ratio, or weight of the testes, epididymides and prostate after DEHP exposure in any of the experimental groups compared to the control. The results are summarized in Table 1.

3.2. Sperm parameters

DEHP administered either during fetal or pubertal development caused a decrease in the sperm concentration in adulthood. We did not observe any changes in sperm viability between the control and

Table 1
Effect of DEHP on AGD, body and reproductive organ weight.

Group	Body weight (g)	AGD (cm)	Testes (g)	Epididymides (g)	Prostate (g)	Seminal vesicles (g)
Control	39.87 ± 0.56	1.62 ± 0.03	0.257 ± 0.006	0.093 ± 0.001	0.149 ± 0.009	0.369 ± 0.012
G	39.06 ± 1.55	1.47 ± 0.07**	0.247 ± 0.028	0.090 ± 0.003	0.147 ± 0.022	0.323 ± 0.045
P	39.77 ± 0.75	1.44 ± 0.02***	0.267 ± 0.011	0.093 ± 0.002	0.158 ± 0.009	0.322 ± 0.018*

Mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001; Control: n = 22; G: n = 5; control, P: n = 18;.(G) gestation period; (P) pubertal period.

Table 2
Sperm parameters.

Group	Sperm concentration (*10^6/mL)	Sperm viability (% live cells)	Sperm morphology (% normal)	Annexin V (% positivity)	HS-8 (% positivity of acrosome)	HS-14 (% positivity of acrosome)
Control	64.50 ± 4.14	88.86 ± 0.59	97.57 ± 0.48	41.86 ± 1.18	93.57 ± 1.13	90.00 ± 2.08
G	37.63 ± 2.75***	88.00 ± 0.45	96.50 ± 0.22*	61.80 ± 2.03***	86.50 ± 0.58***	76.00 ± 1.51***
P	50.93 ± 3.10**	88.60 ± 0.51	96.10 ± 0.43*	44.40 ± 1.50	91.70 ± 0.98	84.60 ± 1.27*

Sperm parameters and immunofluorescence staining. Mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001; Control: n = 22; G: n = 5; control, P: n = 18; (G) gestation period; (P) pubertal period.

experimental groups; however, we observed statistically significant deterioration of sperm morphology in the exposed groups. A higher number of apoptotic sperm (Annexin V positive) was detected in mice exposed during gestation. Acrosomal integrity was impaired in both DEHP-treated groups. The results are summarized in Table 2. We did not observed any apparent differences in sperm motility between samples, as the motility has been checked under the light microscope. Some possible changes in motility parameters cannot be excluded.

3.3. Histology of the testes and TUNEL assay

Testes from the experimental animals did not show any obvious changes in morphology. No pathology in seminiferous tubule morphology was observed in experimental groups compared to the control, and spermatogenesis was not interrupted. Spermatogonia, spermatocytes, elongated spermatids, and sperm in the seminiferous tubule lumen were visible in all experimental groups (Fig. 1A). During cytometrical analysis, we did not observe any significant changes in the number of spermatogonia, Sertoli cells, and pachytene spermatocytes between the control and experimental groups (Fig. 1B). There were no significant differences in the number of TUNEL-positive cells between the control and experimental groups (Fig. 1C).

3.4. Gene expression analysis of the testicular genes

Testicular gene expression was assessed by qRT-PCR, which was performed using either BioMark (germ cell-specific genes; Sertoli cell-specific genes; androgen synthesis and action related genes, apoptosis, oxidative stress and endocrine function related genes) (used primers are summarized in Table S1) or a CFX 384 qPCR cycler (BioRad) (BTB, ES and TBC related genes) (used primers are summarized in Table S2). The DEHP exposure had a stronger effect in mice exposed during puberty

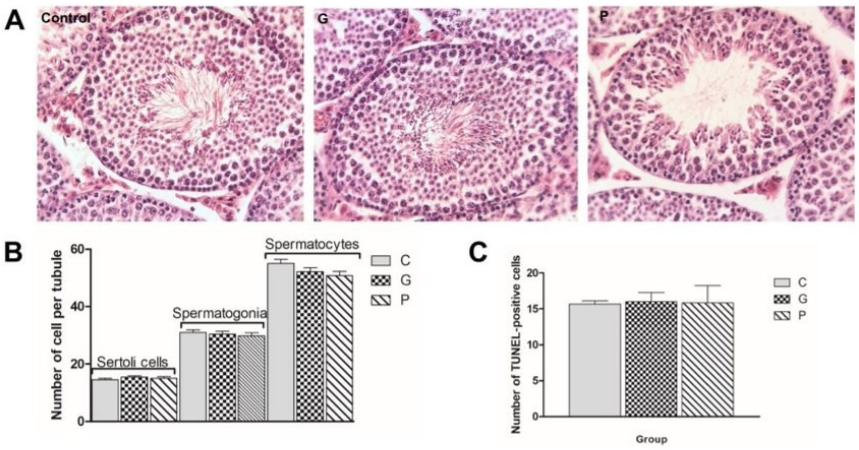


Fig. 1. Histological analysis of testicular tissue and apoptosis in seminiferous tubules after DEHP exposure. (A) Testis sections were stained by hematoxylin (nucleus) and eosin (cytoplasm). Ongoing spermatogenesis was observed in the control group and both experimental groups. There was no pathology in the morphology of seminiferous tubules in any group. Magnification 20 ×. (B) Number of Sertoli cells, spermatogonia, and pachytene spermatocytes in seminiferous tubules in the control and experimental samples. 50 cross-sections of seminiferous tubules were analyzed in five individual animals. (MEAN ± SE). (C) Apoptosis was detected by TUNEL assay and the numbers of TUNEL-positive cells in 20 cross-sections of seminiferous tubules were counted in six testes per group (MEAN ± SE). (C) control; (G) gestation period; (P) pubertal period.

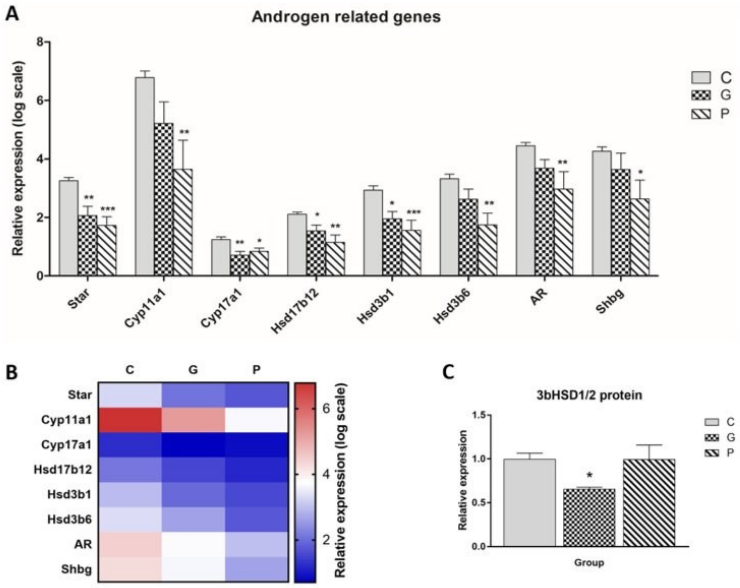


Fig. 2. Expression of genes related to steroidogenesis and androgen action. (A) The graph illustrates the relative mRNA gene expression with ± SEM. Steroidogenesis related genes: *Star, Cyp11a1, Cyp17a1, Hsd17b12, Hsd3b1, Hsd3b6*; androgen action related genes: *Ar, Shbg*. (B) Gene expression analysis outputs represented as a heatmap. (C) Quantification of 3bHSD1/2 protein detection relative to tubulin. The control was set at 1 and the experimental samples were compared to the control. The values represent means ± SEM (n = 6). Significant differences between control (C) and DEHP-treated groups (G, P) were calculated by one-way ANOVA and Dunnett's Multiple Comparison Test for comparison of control and treated groups. *P < 0.05, **P < 0.01, ***P < 0.001. Control: n = 8; G: n = 5; C, P: n = 8.

(group P), persisting in adulthood. In this group, changes in gene expression were observed in the genes whose protein products are involved in androgen synthesis (*Star, Cyp11a1, Cyp17a1, Hsd17b12, Hsd3b1*, and *Hsd3b6*) and androgen responses (*AR, Shbg*). In animals exposed *in utero* (group G), the gene expression of most androgen synthesis related genes was decreased (*Star, Cyp17a1, Hsd17b12, and Hsd3b1*), however expression of genes related to androgen response was not changed. The results are summarized in Fig. 2A,B.

Further, we tested genes specifically expressed in germ cells (*Dazl, Grth, Meig, c-kit, Sycp 1, Sycp 3, Ccan1, Prm1, Prm 2, Tnp 1 and Tnp2*) and Sertoli cells (*Wt1, Gja1, Eps8, Sox8*). Selected germ cells expressed genes showed minor changes in their expression after DEHP exposure. The affected genes were *c-Kit* (downregulated in group P), a gene expressed specifically in spermatogonia, and *Prm1* (downregulated in group G), whose expression is specific for haploid cells. The results are summarized in Fig. 3A,C. The expression of genes specific for Sertoli cells was not changed in group G while decreased expression of genes for *Gja1* and *Eps8* was observed in group P (Fig. 3B,C).

All tested apoptosis-related genes were downregulated in group P (*P21, P53, Bcl2, Fas*) in contrast to group G where the expression of these genes was not affected. The results are summarized in Fig. S1. In case of genes related to oxidative stress (*Sod1, Sod2, Sod3, Gpx4, Cat, Cyct*), we detected significantly decreased expression in the *Cat* gene in group G and Sod 3 gene in group P (Fig. S2).

The last group of genes tested in BioMark was represented by genes related to endocrine functions (*Esr1, Hp, Stx5a, Inha, Fshr, Thra, Lhcgr*). Similarly to previous results, the expression of these genes was affected in animals exposed during puberty (Fig. 4).

Genes related to the formation and integrity of BTB (*Cldn11, Ocln, Tjp1*), ESs and TBCs (*Ncad, Itgb1, Lamc3, Mmp2, Timp2, Tes, Arpc1b, Eea1*) were analyzed in a CFX 384 qPCR cycler. We observed downregulation of *Itgb1, Lamc3, Mmp2* genes in group P and *Mmp2, Arpc1b* genes in group G (Fig. 5). Primary qRT-PCR Cq values are available in Table S3.

3.5. Western blot

To determine whether the observed changes in gene expression at the mRNA level were also observable at the protein level, we performed western blot detection and densitometry. Tubulin detection served as a loading control. A representative western blot is shown in Fig. S3. We observed a significantly decreased expression of 3bHSD1/2, protein involved in androgen synthesis, in the group G (Fig. 2C).

4. Discussion

Numerous studies have shown the adverse effect of DEHP on the male reproductive system [19,20,22,23,31,42–45]; C. [3,24–26]. However, these studies mostly used concentrations much higher than the calculated daily DEHP exposure, and therefore may not fully reflect the real risk of DEHP exposure to human and animals. Interestingly, a long-life impact on male mice reproduction and the most pronounced changes after prenatal exposure to phthalate mixture (including DEHP) have been observed at the lowest (20 µg/kg/day) and highest (500 mg/kg/day) tested doses [46]. This indicates an importance to test EDs also at low concentrations. In our study, the animals were exposed to low dose of DEHP (2.4–3 µg/kg b.w) which is in range of DEHP daily human intake (1–4.2 µg/kg b.w). We determine the exposure concentration based on volume of water drank on average per day. We were aware that precise exposure could have been done by injection, stomach tube or alternative methods of applying drugs. Unfortunately, these methods are very stressful for animals and thus affect the fertility, which was the major target of the study, therefore we did not use any of them. The tested dose is below the NOAEL for DEHP [31]. Calculated half-life of DEHP is 2.33 days in rats [10] thus the accumulation of DEHP and/or its metabolites is predicted in our mice model. Moreover, DEHP and its metabolites can pass into breast milk [8,10,14] therefore mice treated between GD 10 and GD 20 were likely additionally exposed to DEHP shortly after birth *via* breastfeeding. During the experiments, male siblings were housed together (maximum 4 animals per cage). We did not observe any behavioural hierarchy or fights between siblings.

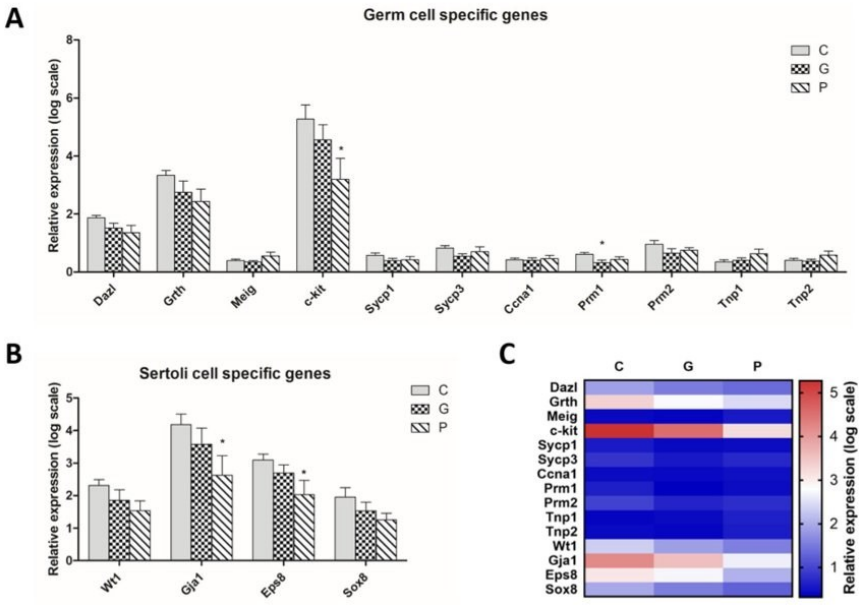


Fig. 3. Quantitative RT-PCR analysis of genes expressed in testicular germ cells and Sertoli cells. The graphs illustrate the relative gene expression with ± SEM. (A) Genes expressed in multiple germ cell types (*Dazl, Grth, Meig1*), spermatogonia (*c-Kit)*, during meiosis (*Sycp1, Sycp3, Ccna1*) and in haploid germ cells (*Prm1, Prm2, Tnp1, Tnp2*). (B) Genes expressed in Sertoli cells. (C) Gene expression analysis outputs represented as a heatmap. Significant differences between control (C) and DEHP-treated groups (G, P) were calculated by one-way ANOVA and Dunnett's Multiple Comparison Test for comparison of control and treated groups. *P < 0.05; Control: n = 8; G: n = 5; C, P: n = 8.

Therefore, we can exclude that an individual mouse exposure could be affected by competitive behaviour.

The first phenotypic change we observed, was shortening of AGD in both experimental groups and reduced weight of seminal vesicles in group P. In group G, we also observed a decreasing trend in the weight of seminal vesicles. As both parameters are androgen dependent [16,47], these results suggest a disruption of androgen signalling during MPW or puberty in our treated mice. This may be caused by an altered level of androgens or alternatively by an anti-androgenic effect of DEHP. Changes in AGD and the weight of seminal vesicles were also detected in other studies performed on rats (K [19,48].) and mice [49]. The reduced AGD was also observed in males prenatally exposed to phthalates [50] which indicates that phthalates could affect humans in the same way DEHP affected our experimental mice.

In the next step, we examined sperm quality as one of the main reproductive parameters. We observed a decrease of sperm concentration by 43 % (group G) and by 22 % (group P), increase of apoptotic sperm (group G) and slightly disrupted morphology and acrosome integrity in all treated animals. It should be mentioned that despite the relatively high decrease in sperm concentration in DEHP treated animals, these concentrations still reached the limit sufficient for mouse reproduction [38,51]. Decreased sperm concentration, motility and impaired sperm morphology have also been detected in mice treated with a higher dose of DEHP [3]. Postnatal DEHP exposure at the concentration similar to that used in our study was reported to disrupt sperm morphology and higher concentrations affected sperm count [22]. Surprisingly, increased sperm count has been detected in mice after chronic DEHP exposure [52]. The discrepancies could arise from

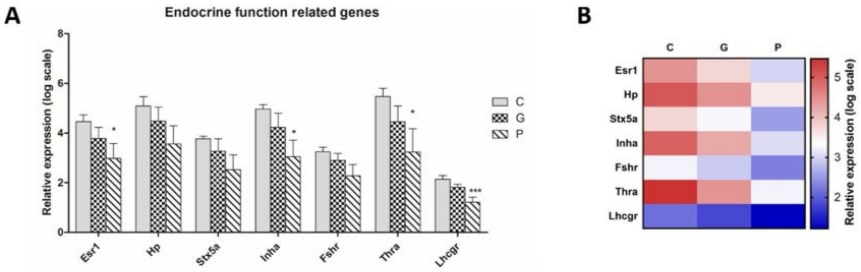


Fig. 4. Quantitative RT-PCR analysis of genes related to endocrine function. (A) The graph illustrates the relative gene expression with ± SEM. (B) Gene expression analysis outputs represented as a heatmap. Significant differences between control (C) and DEHP-treated groups (G, P) were calculated by one-way ANOVA and Dunnett's Multiple Comparison Test for comparison of control and treated groups. *P < 0.05; Control: n = 8; G: n = 5; C, P: n = 8.

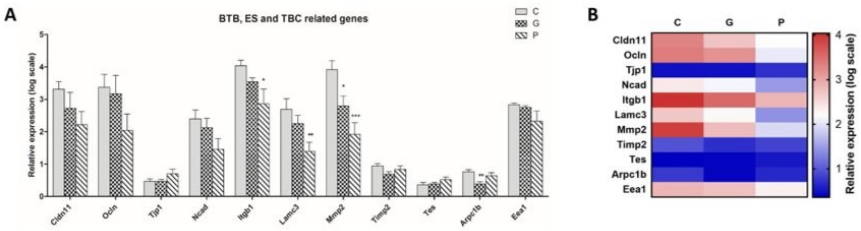


Fig. 5. Quantitative RT-PCR analysis of genes related to the blood-testis barrier, ectoplasmic specialization, and tubulobulbar complex. (A) The graph illustrates the relative gene expression with ± SEM. Blood-testis barrier related genes (BTB): *Cldn11, Ocln, Tjp1*; ectoplasmic specialization (ES) related genes: *Ncad, Itgb1, Lamc3, Mmp2, Timp2, Tes;* tubulobulbar complex (TBC) related genes: *Arpc1b,* Eea1. (B) Gene expression analysis outputs represented as a heatmap. Significant differences between control (C) and DEHP-treated groups (G, P) were calculated by one-way ANOVA and Dunnett's Multiple Comparison Test for comparison of control and treated groups. *P < 0.05, **P < 0.01, ***P < 0.001; Control: n = 8; G: n = 5; C, P: n = 8.

different exposure periods, used DEHP concentrations and age of analyzed mice.

To reveal the mechanism of observed DEHP action, we analyzed the gene expression and histology of testes. The examination of testicular morphology did not reveal any impairment of testicular architecture or cellular content. Analysis of germ cell-specific gene expression once again suggested that in our study, there was no disturbance of spermatogenesis in correlations with no found testicular histopathological changes in 60 days old mice prenatally treated with high dose of DEHP. However, severe pathological abnormalities along with increased germ cell apoptosis were found in these mice at 22 months of age [3]. One could speculate whether an altered testicular morphology would be found if the older mice were analyzed.

The lower epididymal sperm count may be explained by an increase in germ cell apoptosis [38]. DEHP exposure has been shown to cause oxidative stress and apoptosis in testicular cells [26,53]. However, this does not seem to be the case in our study, as testicular cell apoptosis has not been changed in experimental mice. Also, the gene expression results did not indicate that DEHP exposure would cause oxidative stress. However, we detected a significantly increased number of apoptotic epididymal sperm in animals exposed *in utero*. We hypothesize that spermiogenesis or epidydimal maturation might be also disrupted in these animals which can cause the apoptosis of epidydimal sperm and further contribute to a decrease of sperm concentration.

Our results from evaluation of classical reproductive parameters suggested that DEHP disrupts the androgen signalling thus we tested a group of genes which are involved in steroidogenesis and testosterone signalling. Similar to observed results with AGD and the weight of seminal vesicles, DEHP had a higher effect in animals exposed during puberty where all tested genes were significantly downregulated. The changes in steroidogenesis after DEHP or its metabolite treatment were also observed in previous studies in both males and females [42–44]. Unfortunately, we were not able to determine the blood testosterone concentration of exposed animals. The testosterone serum level data collection was ongoing through the experiments, but we were facing several technical issues in obtaining the consistent data, therefore they were not included to the study.

Sertoli cells (SCs) are crucial for proper sperm production, and their proliferation and maturation is under the control of androgen signalling [54]. Disturbance of SC differentiation regulating pathway with subsequent loss of SCs in the postnatal mice offspring have been found after *in utero* exposure to high doses of DEHP [55]. We did not observe an altered number of SCs in exposed animals. In order to reveal possible disruption of SC function, we tested the expression of several Sertoli cell genes and we detected a decreased expression of *Gja1* and *Eps8* genes in animals exposed postnatally. GJA1 is a predominant gap junction protein in testis, participates in the formation and regulation of the blood-testis barrier (BTB), and its expression in Sertoli cells is necessary to maintain fertility [56]. EPS8 is a signalling molecule at apical ectoplasmic specialization (a junctional complex formed between SCs and spermatids). Interestingly, the apical ectoplasmic specialization can be disrupted by suppression of endogenous testosterone [57]. Androgen signalling in Sertoli cells is also known to regulate permeability of the BTB [58]. Due to a decreased expression of steroidogenic genes, *AR, Gja1,* and *Eps8*, we assumed that the interaction between SC−SC and SC-germ cells may be affected, and thus we complemented our study of genes whose protein products are involved in the formation and regulation of BTB, ectoplasmic specializations (ESs), and tubulobulbar complexes (TBCs). Little functional damage of BTB after DEHP treatment at doses similar to ours was found in the study on testicular immune microenvironment [45]. We did not confirm the negative effect of DEHP on BTB, however we did not perform a functional study and the exposure time of mice was different. The effect of DEHP on TBC was negligible, however, significantly decreased expression was found in two genes related to ES *(Itgb1, Lamc3*) in group P. Reduced expression of ITGB1 and LAMC3 in apical ESs was also observed in 28-day-old mice after a single dose of MEHP (1 g/kg) [59]. ITGB1 and LAMC3 are part of protein complexes constituting the apical ES, the only anchoring device for spermatids from step 8 [60] and their reduced expression may indicate impaired function of ES. One (*Mmp2*) of two tested genes (*Mmp2, Timp2*) whose protein products regulate apical ES degeneration and subsequently remodelling of BTB [60] was downregulated in both experimental groups, which further supports the hypothesis that the function of ES might have been compromised. Expression of most of these tested genes is testosterone regulated, and thus changes in their expression may be the secondary effect of reduced testosterone production indicated from reduced AGD, lower weight of seminal vesicles, and decreased expression of steroidogenic genes.

The last group of the tested genes was related to endocrine regulation. We found reduced expression of *Lhcgr* gene in the group P, which may indicate reduced LH signalling. As LH signalling is necessary to stimulate androgen production in Leydig cells [61], the reduced *Lhcgr* expression may be a cause of lower steroidogenesis, which is consistent with our result from steroidogenic gene expression. Decreased expression of *Esr1, Thra* and *Inha* in group P indicates that DEHP treatment may also target the receptors for estrogens, thyroid hormones and inhibins. In recent years, there has been growing evidence that in addition to testosterone, estrogens also play an important role in regulation of spermatogenesis and male fertility [62]. Thyroid hormones impact testicular development, and their abnormal profile affects the semen quality and male fertility [63]. Inhibin alpha together with inhibin beta form a glycoprotein hormone inhibin, which is produced by adult Sertoli cells and suppresses synthesis and release of FSH from the pituitary [64,65].

In general, we observed a similar outcome in all the analyzed parameters for both DEHP experimental groups, even though, *in utero* exposed animals displayed a profound negative effect on sperm concentration and quality. On the other hand, in animals exposed during

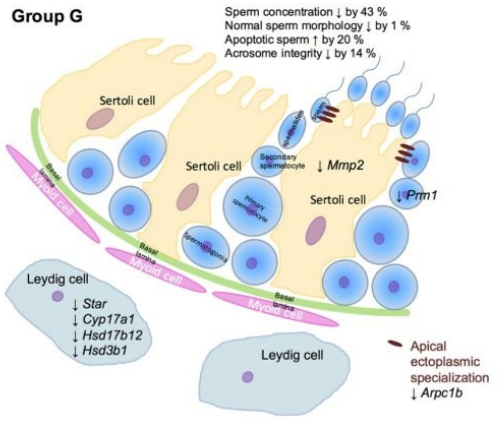


Fig. 6. Summary of results obtained from analysis of prenatally DEHP exposed male mice (group G). This picture is showing drawing of testicular tissue and different cell population present in testes. Data from analysis of gene expression and sperm parameters in animals are shown in respective cell types.

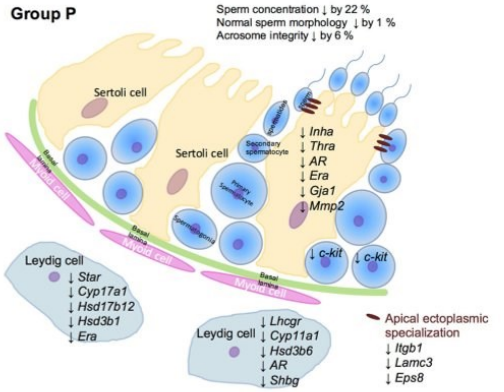


Fig. 7. Summary of results obtained from analysis of postnatally DEHP exposed male mice (group P). This picture is showing drawing of testicular tissue and different cell population present in testes. Data from analysis of gene expression and sperm parameters in animals are shown in respective cell types.

puberty we report on significant changes in the level of gene expression. However, in both DEHP treated groups, there was not a noticeable alteration in spermatogenesis based on measured parameters. We hypothesize that observed lower epididymal sperm concentration and lower sperm quality might be caused by DEHP interference with androgen signalling. Based on our mRNA analysis we propose that altered androgen signaling may cause disturbance of ectoplasmic specialization. For better understanding of our findings we review our results in summarizing Figs. 6 and 7.

5. Conclusion

Our study showed that a low concentration of DEHP, equivalent to a human daily intake, is able to negatively influence sperm parameters and testicular gene expression of mice *in vivo*. It is alarming that, alterations caused by DEHP exposure *in utero* were observable in adult males. In treated groups, we detected decreased sperm concentration and impairment of sperm quality such as morphology, acrosome integrity and increase of apoptotic spermatozoa. We speculate that DEHP interferes with Leydig and Sertoli cell maturation during sensitive developmental periods, which triggers epigenetic changes that in turn lead to lower steroidogenic activity, compromised the function of the endocrine system, and damaged SC-germ cell interaction in adulthood. Improper adhesion and communication between SC-germ cells may result in decreased spermatogenesis output and the decreased sperm concentration may be further caused by the DEHP interference with epididymal maturation. Further study of protein expression and localization is required to discover a mechanism of the DEHP effect on the ectoplasmic specializations. Moreover, a study of the DEHP exposure with subsequent testosterone administration would help to clarify, whether the observed changes are directly due to DEHP exposure or whether they are related to decreased testosterone signalling.

Authors' contributions

PD, EZ and JP designed the study; LD performed the statistical analysis of the phenotypic and gene expression data; FE performed the *in vivo* study; EV and PD performed the PCR and western blot analysis; AK analyzed the sperm quality; VK, LL and EZ performed the BIOMARK analysis; PD, EZ, KK and JP wrote and revised the paper. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.reprotox.2020.06.014.

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