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Effect of selected endocrine disruptors on the male mouse reproductive system *in vivo*

Vliv vybraných endokrinních disruptorů na reprodukční systém myších samců *in vivo*

Ph.D. Thesis

Supervisor:

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Prague, 2015

Statement:

I hereby declare that I have completed this final thesis independently and that I have included all cited literature and used sources of information. Neither the work as a whole nor any of its substantial parts have been previously submitted to achieve a different or similar academic degree.

Prague, ...

Signed:

Eva Žatecká

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Abstract

In our environment there are many compounds which can negatively influence humans and wildlife. Every day, a vast number of environmental pollutants are released into our environment and there is no way to avoid their exposure. Some of these compounds can even mimic endogenous hormones and interfere with our endocrine system (so called endocrine disruptors), which is the key regulatory system controlling almost all physiological processes in human and animal bodies. Also the reproductive system is largely regulated by various hormones, and their proper function is crucial for gamete formation, fertilization and embryo development. Environmental pollutants are therefore considered as one of the possible causes of increased infertility in human population. This prompted us to study the effect of two endocrine disruptors (tetrabromobisphenol A – TBBPA, and zearalenone – ZEA) on the male mouse reproductive system *in vivo*.

According to our results, TBBPA is able to induce apoptosis as well as changes in the expression of selected testicular genes and sperm protamination. Our results also suggest that permanent exposure to TBBPA slightly enhances its effect in the next generation, depending on whether the parents have been affected or not. We hypothesized that differential protamination of the sperm DNA may be one of the possible mechanisms of trans-generational transmission of the pathological phenotypes induced by environmental pollutants.

Results from our next study have shown that ZEA is able to negatively influence the sperm quality, mainly sperm concentration and morphology. Based on our results from gene expression analysis we assumed that the most affected cells are spermatogonia and meiotic germ cells. Our results have also shown that the lower dose of ZEA had a greater effect on exposed animals.

Finally, in the third part of the presented work we analyzed expression of selected genes in specimens from testicular biopsies of azoospermic patients who underwent TESE. Our results showed that gene expression analysis can be an additional and useful tool for assessing the most suitable procedure for each patient.

Souhrn

Každý den je do našeho prostředí uvolňováno ohromné množství tzv. polutantů životního prostředí, které mohou negativně ovlivnit naše zdraví. Některé z těchto sloučenin jsou hormonálně aktivní látky (tzv. endokrinní disruptory), které mohou interferovat s naším hormonálním systémem. Hormonální systém ovlivňuje správnou funkci mnoha fyziologických procesů a je jeden z nejdůležitějších regulačních systémů v organismu. I reprodukční systém je do značné míry regulován různými hormony a jejich správná funkce je zásadní pro tvorbu gamet, oplodnění a vývoj embrya. Proto znečištění životního prostředí je považováno za jednu z možných příčin zvýšené neplodnosti v lidské populaci. V našich studiích jsme se proto rozhodli studovat vliv dvou endokrinních disruptorů (tetrabrombisfenol A - TBBPA a zearalenon - ZEA) na samčí reprodukční systém myší *in vivo*.

Podle našich výsledků je TBBPA schopen indukovat apoptózu testikulárních buněk, stejně jako změny v expresi vybraných testikulárních genů a protaminaci spermií. Dále naše výsledky naznačují, že při kontinuální expozici TBBPA dochází k akumulaci jeho negativního vlivu v další generaci v závislosti na tom, zda rodiče byli nebo nebyli ovlivněni. Jedním z možných mechanismů trans-generačního přenosu by mohly být pozorované změny v protaminaci spermií.

Výsledky z naší další studie ukázaly, že ZEA má negativní vliv na kvalitu spermií, zejména na koncentraci a morfologii. Výsledky z analýzy genové exprese naznačují, že mezi nejvíce ovlivněné buňky patří spermatogonie a meiotické zárodečné buňky. Naše výsledky rovněž ukázaly, že nižší dávka ZEA má výraznější efekt na testované reprodukční parametry než dávka vysoká.

V naší poslední studii jsme analyzovali expresi vybraných genů ve vzorcích z testikulárních biopsií pacientů s azoospermií, kteří podstoupili TESE. Naše výsledky ukazují, že analýza genové exprese může být užitečnou diagnostickou metodou, která pomůže k výběru nejvhodnějšího postupu pro každého pacienta.

Abbreviations

Acr	acrosin gene	protein coding gene
Actb	β-actin	cytoskeleton
Anapc11	anaphase-promoting complex subunit 11	expressed during meiosis
Ar	androgen receptor	testosterone signaling
ART	assisted reproductive technology	
Bax	Bcl2-associated X protein	
Bcl-2	B-cell lymphoma	protein family
BFRs	Brominated flame retardants	
bw	body weight	
Ca^{2+}	Calcium ion	
Ccna1	cyclin-A1	spermatocyte marker
Ccnd1	cyclin D1	cell cycle
cDNA	complementary DNA	
c-kit	mast/stem cell growth factor receptor	cytokine receptor
Cq	quantification cycle	qPCR metric
Crem	cAMP responsive element modulator	
Ctds	cathepsin D	
DAPI	4',6-diamidino-2-phenylindole	fluorescent stain (DNA)
DNA	deoxyribonucleic acid	nucleic acid
Dnmt1	DNA (cytosine-5)-methyltransferase	DNA methyltransferase
dUTP	deoxyuridine 5'-triphosphate	nucleotide
ED(s)	endocrine disruptor(s)	
Eps8	epidermal growth factor receptor kinase substrate 8	
ER(s)	estrogen receptor(s)	receptor
F1	first generation	
F2	second generation	
Fkbp5	FK506 binding protein 5	
FSH	follicle-stimulating hormone	hormone (pituitary)
Fshr	follicle-stimulating hormone receptor	
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	enzyme-glycolysis
GH3	rat pituitary tumor cell line	
GnRH	gonadotropin-releasing hormone	hormone (hypothalamic)
Grth	gonadotropin-regulated testicular helicase	RNA helicase
HS	hypospermatogenesis	
Hsps	heat shock proteins	protein chaperones
H3K4me2	histone H3 dimethylated at lysine 4	histone mark
H3K27me3	histone H3 trimethylated at lysine 27	histone mark
Icap1	integrin beta-1-binding protein 1	Sertoli cell marker
ICSI	intracytoplasmic sperm injection	assisted reproductive technology
Igfbp5	insulin-like growth factor binding protein 5	
IVF	*in vitro* fertilization	assisted reproductive technology

Kdm4a	lysine-specific demethylase 4A	histone demethylase
Mas1	mas proto-oncogene	
MA	maturation arrest	
MCF-7	human breast adenocarcinoma cell line	
Meig 1	meiosis expressed gene 1	spermatocyte marker
MESA	microscopic epididymal sperm aspiration	assisted reproductive technology
MND1/GAJ	meiotic nuclear division protein	expressed prior meiosis
P	protamines	sperm nuclear proteins
P1	protamine 1	sperm nuclear protein
P2	protamine 2	sperm nuclear protein
p21	cyclin-dependent kinase inhibitor 1	cell cycle
p53	tumor suppressive gene	cell cycle
Prm1	protamine 1	gene
PCR	polymerase chain reaction	DNA amplification method
qPCR	quantitative polymerase chain reaction	DNA amplification method
RNA	ribonucleic acid	nucleic acid
SCO	Sertoli cell only syndrome	
Sik1	serine/threonine-protein kinase	protein kinase
Sox9	sex determining region Y-box 9	Sertoli cell marker
Spata2	spermatogenesis-associated protein 2	FSH-dependent
Sycp1	synaptonemal complex protein 1	spermatocytes specific
Sycp3	synaptonemal complex protein 3	spermatocytes specific
T4	thyroxine	thyroid hormone
Tff1	trefoil factor 1	
TBBPA	tetrabromobisphenol A	flame retardant
TESE	testicular sperm extraction	assisted reproductive technology
TH	thyroid hormone	hormone
TNP1	transition protein 1	spermatid marker
TNP2	transition protein 2	spermatid marker
TR	thyroid receptor	receptor
TTR	transthyretin	TH transport protein
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling	
Vegfa	vascular endothelial growth factor A	growth factor
Wt1	Wilms tumor protein	Sertoli cell marker
ZEA	zearalenone	product of Fusarium fungi
α-ZOL	alpha zearalenol	metabolite of ZEA
β-ZOL	beta zearalenol	metabolite of ZEA

1. Introduction

In recent years, infertility became a common problem in human population and therefore an important topic for scientific and medical research. According to the World Health Organization, 15 % of couples in reproductive age suffer from infertility problems, and up to 50 % of cases are caused by the male (Hirsh, 2003; Poongothai et al., 2009). One of the most common causes of male infertility is severely decreased sperm quality. Several studies have shown that sperm quality, in particular sperm concentration, has decreased globally over the past 60 years (Carlsen et al., 1992; Swan et al., 1997, 2000). These data generated much controversy because of the geographical and ethnical variation or different study designs. However, it also attracted attention of physicians and researchers to the problem of male infertility and its causes. Causes of the decrease in sperm quality and subsequent infertility problems can be numerous, such as stress, older age of parents, unhealthy lifestyle or smoking, and last but not least the poor quality of our environment.

Many different pollutants and endocrine disruptors appear in our environment. They are released from factories or various products and can get into the water, food or air and influence our health. Therefore, it is necessary to reveal which of these pollutants are dangerous to our health to eliminate their usage. The field of science that studies the harmful effects of these pollutants is called environmental toxicology. Reproductive toxicology is particularly focused on the effect of these pollutants on reproduction. For this purpose, various *in vitro* and *in vivo* models are used. The main objective of reproductive-toxicological studies is the effect of various pollutants especially on the endocrine system, spermatogenesis, and subsequently the sperm quality. Every year all published results are collected and the potential harmful effect of the tested compounds is assessed. These studies thus provide important information that can lead to restriction of usage of the harmful substance.

1.1. Spermatogenesis

The following part will be dedicated to the process of spermatogenesis as one of the most important processes in the male reproduction. Proper spermatogenesis is absolutely

crucial for production of functional sperm, and disruption of this process is very often the cause of male infertility.

Spermatogenesis takes place in seminiferous tubules, which are long and convoluted structures inside the testis. Seminiferous tubules consist of various types of germ cells that are surrounded by supporting Sertoli cells. The interstitial space between particular seminiferous tubules consists of Leydig and myoid cells. All these cell types help create the environment necessary for proper spermatogenesis.

The process of spermatogenesis begins with a series of mitotic divisions of primordial germ cells, which give rise to spermatogonia (types A1 - A4). Type A spermatogonia still retain the ability of stem cells, which means that they are able to divide and provide a constant supply of type A spermatogonia or to differentiate into type B spermatogonia. Type B spermatogonia are already determined for sperm differentiation and they lack the properties of stem cells. These cells divide mitotically and give rise to primary spermatocytes. Primary spermatocytes then enter the first meiotic division and form secondary spermatocytes, which subsequently complete the second meiotic division and produce haploid round spermatids (Dym, 1994) (Fig. 1).

Meiosis is a special type of cell division typical of sexual reproduction during which the number of sets of homologous chromosomes in a diploid cell is reduced to a half. A cell with thereby halved chromosome number is referred to as a haploid cell. Meiosis proceeds in several steps and each step is characterized by certain actions. The first phase is called prophase I and it is characteristic by pairing of homologous chromosomes and subsequent homolog recombination during which the sister chromatids exchange part of their genetic information (so called crossing over) (Stewart and Dawson, 2008). During this phase the protein structure called synaptonemal complex is formed between homologous chromosomes and mediates the chromosome pairing and recombination. Prophase I is followed by metaphase I, when the meiotic spindle is formed and spindle fibers are attached to chromosome kinetochores. During anaphase I, the kinetochore fibers shorten and pull the homologous chromosomes apart (each chromosome consisting of two chromatids). Finally, during telophase I, the chromosomes arrive to the poles, the nuclear envelope is re-established and cytokinesis occurs (Bisig et al., 2012). After the first meiotic

division, each daughter cell has half the number of chromosomes consisting of a pair of chromatids. The second meiotic division is mechanically similar to mitosis. The spindle is attached to the chromosome kinetochores and separation of sister chromatids occurs, resulting in cells with haploid genome. It is important to realize that during spermatogenesis the cell division is not complete, but the cells remain connected with cytoplasmic bridges. These bridges facilitate communication between the cells and synchronizing the entire process (Dym and Fawcett, 1971).

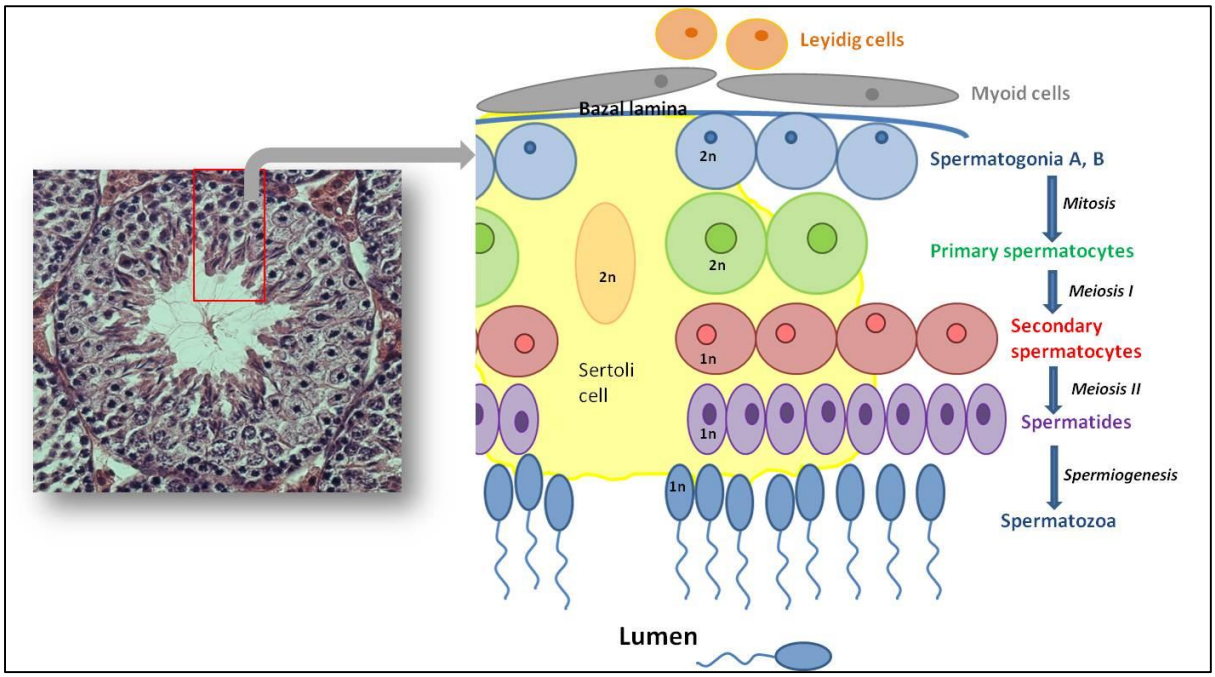

Fig. 1. *Scheme of the seminiferous tubule with ongoing spermatogenesis and particular cell types present in testicular tissue*

Mammalian spermatogenesis is a highly coordinated process and throughout all the stages, the developing germ cells are attached to the Sertoli cells via specialized cell junctions (Lui and Cheng, 2012). In adulthood, Sertoli cells support spermatogenesis, but it is not the only function they have. Sertoli cells also play an important role during testis formation in fetal and early postnatal life (Sharpe et al., 2003). At puberty the role of Sertoli cells switches to supporting germ-cell differentiation and they also form the blood-testis barrier. The blood-testis barrier is created by adjacent Sertoli cells near the basal lamina and divides the seminiferous epithelium into the basal and apical compartments (Lui and Cheng, 2012). The maturation of Sertoli cells is controlled by hormones; in particular follicle-stimulating hormone (FSH) and thyroid hormone (TH). TH inhibits Sertoli cell

proliferation and stimulates their functional maturation, while FSH functions as a pro-proliferative factor (Sharpe et al., 2003). The number of Sertoli cells determines the number of germ cells that can be produced through spermatogenesis and hence determines the efficiency of sperm production. After the maturation during puberty, Sertoli cells are considered as non-proliferative; however, some studies have shown that Sertoli cells are able to regain their proliferative ability even in adulthood (Ahmed et al., 2009; Tarulli et al., 2012).

1.1.1. Spermiogenesis

Spermiogenesis, sometimes also called spermateliosis, is the final stage of spermatogenesis when the transformation of round spermatids into differentiated spermatozoa occurs. It is a metamorphosis process, which involves condensation of the nuclei, formation of flagellar and acrosomal structures, and loss of a significant amount of cytoplasm. During this process many organelles such as the nucleus, endoplasmatic reticulum, Golgi apparatus, mitochondria, and centriole undergo structural and functional changes that are necessary for proper function of spermatozoa (de Kretser et al., 1998).

In early stage of spermiogenesis, the Golgi apparatus is transformed into the acrosome vesicle, which is placed over the anterior half of the sperm head. Acrosome is quite similar to a cellular lysosome; it is a sack-like structure composed of inner and outer acrosomal membrane and contains various digestive enzymes (e.g. hyaluronidase or acrosin). Acrosome plays an important role during fertilization, in particular during the passage of sperm through oocytes' external covers and sperm-oocyte binding. Another step is formation of the flagella structures, when the future axoneme grows out of one centriole and the flagellum is extended into the lumen of seminiferous tubules. During the flagellum formation, mitochondria align along the elongating flagella and are placed in the flagellar midpiece, where they form a ring around the axoneme (Abou-Haila and Tulsiani, 2000).

During the last stage of spermiogenesis, condensation of the sperm nucleus takes place. This step-wise process includes disassembly of the nucleosome structure, replacement of histones by transition proteins (TNPs) and finally by protamines. The mechanisms controlling these processes are not fully understood; however, it is known that before the

nucleosome disassembly occurs, there is an extensive synthesis of histone variants (some of them testis specific) followed by stage-specific post-translational histone modification (mainly acetylation) (Oliva, 2006). Govin et al. (Govin et al., 2004) suggest that in this way, a new testis-specific "histone code" is formed, which could direct the histone-protamine transition. Moreover, acetylation induces changes of histone conformation and charge, resulting in a decrease of DNA-histone binding and thus facilitating the nucleosome disassembly and histone replacement (Oliva et al., 1987). The importance of histone acetylation during spermiogenesis has been shown in the study by Sonnack et al. (Sonnack et al., 2002), who demonstrated a link between the decrease of histone acetylation and incorrect histone-to-protamine exchange.

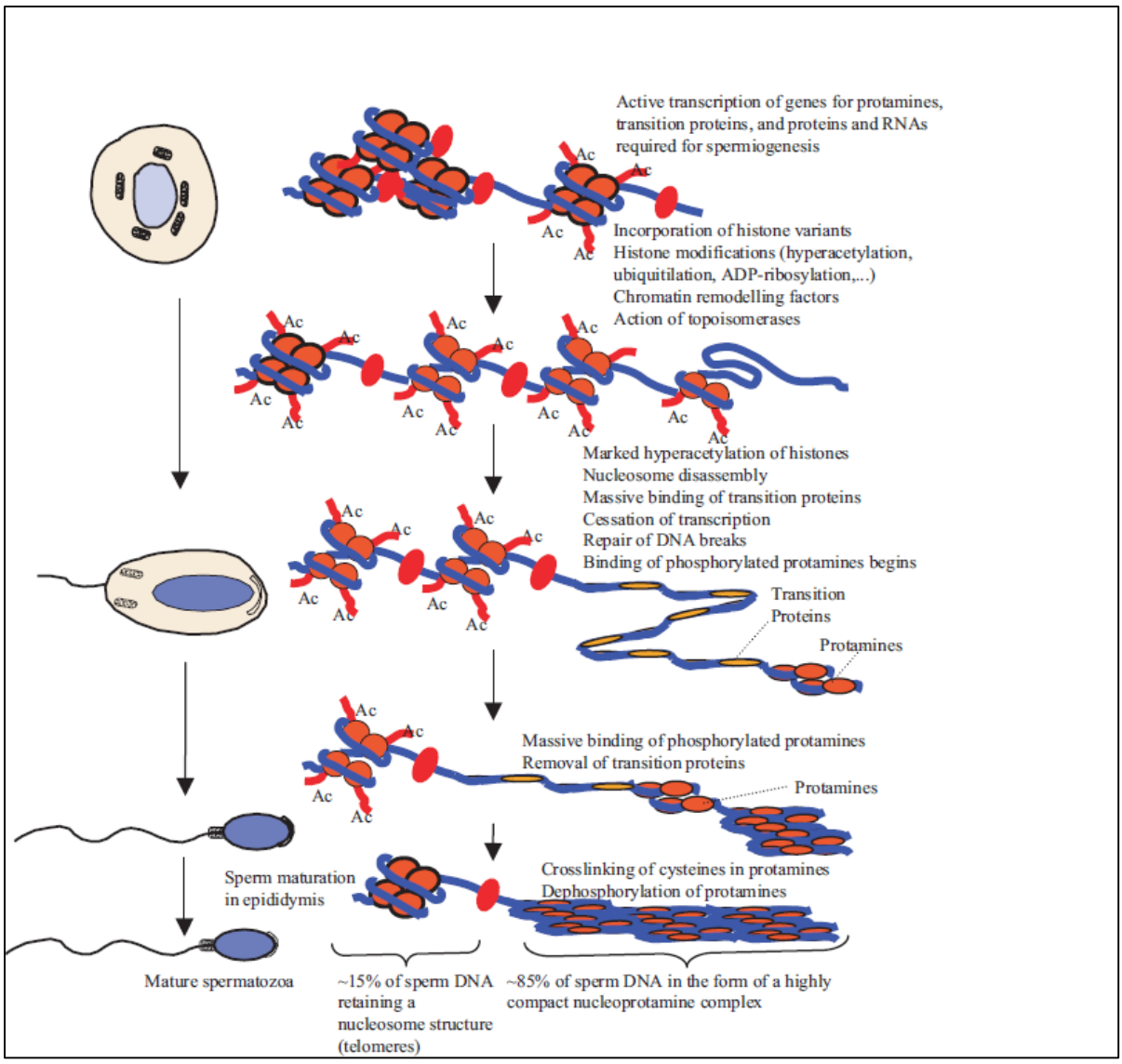

Fig. 2. *Diagram of histone to protamine transition during spermiogenesis (Oliva, 2006)*

Nucleosome disassembly is followed by massive binding of TNPs, which serve as alignment factors for DNA strands. Furthermore, TNP1 is also involved in the repair of DNA breaks (Zhao et al., 2004). Transition proteins are subsequently replaced by protamines. It is known that protamines are phosphorylated shortly after translation and that their phosphorylation is essential for proper protamine binding to DNA. Once protamines are bound to DNA, they are dephosphorylated and a highly compact nucleoprotamine complex is formed (Carrell et al., 2007) (Fig. 2). Once the sperm DNA is tightly packed with protamines, the overall transcription is terminated and the sperm nucleus remains transcriptionally silenced.

1.1.2. Protamines

Protamines are the most abundant sperm nuclear proteins. They are small highly basic proteins with high content of arginine. They also contain cysteines, which enable them to form disulfide bridges that further stabilize the nucleoprotamine complex (Balhorn, 2007). Protamine transcription and translation takes place during the post-meiotic phase of spermatogenesis. Protamine mRNA is present in round spermatids; however, the translation is delayed and is observed in elongated spermatids (Steger, 1999). Protamines pack DNA in a different manner compared to histones. Protamines coil the DNA into large toroidal structures of about 50 thousand base-pairs, which leads to 10-fold compaction of the male genome (Allen et al., 1993). In mature human spermatozoa, about 85 % of histones are replaced by protamines. In mouse species the replacement is even more pronounced and about 99 % of histones are replaced by protamines (Brunner et al., 2014).

In mammals, there are two types of protamines – protamine 1 (P1) and protamine 2 (P2). Protamine 1 is present in the sperm nucleus of all studied mammalian species, whereas protamine 2 is only present in some of them, including human and mouse (Corzett et al., 2002). The relative proportion of the two protamines differs depending on the genus, but it is consistent within the same species. In normal human spermatozoa, the P1 to P2 ratio (P1/P2) is approximately 1.0 (Carrell and Liu, 2001). In mature mouse spermatozoa the content of P2 is higher and the P1/P2 ratio is around 0.4 (Corzett et al., 2002).

One of the main functions of protamines is condensation of the paternal genome. Before fertilization, sperm must undergo the journey through the female genital tract, where it could be potentially exposed to various nucleases and mutagens. However, tightly condensed DNA is inaccessible to these substances, and thus the sperm genetic information is protected against damage. Also condensation of DNA results in a compact and hydrodynamic nucleus, which is linked with the shape of the sperm head. Indeed, it has been shown that protamine deficiency is linked with abnormal sperm head morphology (Cho et al., 2001).

Many studies have shown a link between alterations of protamine content or protamine ratio and male infertility. Changes in normal P1/P2 ratio were observed in patients having problems with conceiving; however, no changes in P1/P2 were observed in fertile donors (Carrell and Liu, 2001; Mengual et al., 2003; Oliva, 2006; Jodar and Oliva, 2014). Other studies have shown a correlation of abnormal protamination with increased sperm DNA fragmentation, which resulted in lower fertilization rates, poor embryo quality and reduced pregnancy rates (Khara et al., 1997; Aoki et al., 2005; Simone et al., 2012). Differences in protamine content or ratio may have various causes such as changes in protamine transcription, altered translation of the transcript, or problems with post-translational modifications.

The sperm, besides carrying the paternal DNA, also transmits epigenetic information, e.g. DNA methylation or activity of small RNAs, which could be transmitted to the oocyte and influence the progeny (Stouder and Paoloni-Giacobino, 2011; Kiani et al., 2013; Liebers et al., 2014). However, DNA methylation or small RNAs are not the only type of epigenetic information that can be delivered by sperm to the oocyte. There is growing evidence that the distribution of genes in the regions associated with protamines or histones in the sperm is not random (Li et al., 2008; van der Heijden et al., 2008). Hammound et al. (Hammoud et al., 2009) were able to identify sperm DNA which remains associated with histones and they have shown that this DNA contains loci important for early development, including imprinted gene clusters, miRNA clusters, and promoters of developmental transcription and signaling factors. Moreover, they have demonstrated the presence of activating (H3K4me2) and repressive histone modifications (H3K27me3) localized to promoters of genes encoding transcription factors significant for embryo development.

The activating and repressive histone marks on distinct promoters have also been detected in mouse sperm (Brykczynska et al., 2010). Furthermore, recently, changes in histone distribution in sperm DNA of infertile patients compared to fertile donors have been observed (Hammoud et al., 2011). Taken together, epigenetic marking in sperm seems to be more extensive than previously thought and histone/protamine distribution is an important part of it.

The study by Cho et al. (Cho et al., 2001) nicely demonstrated the importance of protamines for proper sperm function and embryo development in mice. Authors have shown that both protamines are essential and that a decrease in the amount of either protamine impairs nuclear formation and leads to altered spermatogenesis and male infertility.

1.1.3. Genetic and hormonal control of spermatogenesis

As mentioned above, spermatogenesis involves precise regulation of different cellular events that take place in the seminiferous epithelium composed of Sertoli cells and developing germ cells, surrounded by Leydig and myoid cells. These events require precise co-ordination, which occurs on three regulatory levels – **extrinsic**, **interactive** and **intrinsic** regulation (Eddy, 2002).

Intrinsic control is provided by evolutionarily conserved genetic program that determines when specific genes are expressed. During spermatogenesis, various germ cell-specific transcripts are produced and many of them are transcribed only during a specific stage of male germ cell development (Fig. 3). These transcripts can be produced in several ways: a) they can be products of genes that are germ cell-specific homologs of somatic genes; b) others can be products of unique genes; c) some of them can be products of alternative splicing (Eddy and O'Brien, 1998). The development of male germ cells includes processes that do not occur elsewhere, such as meiosis, genetic recombination, formation of the acrosome and flagellum, and remodeling and condensation of chromatin. All these processes are highly ordered and require specific proteins and well-coordinated timing – the regulation of gene expression is thus crucial for proper spermatogenesis.

Interactive regulation represents communication of neighboring cells that influence each other. In this system, the key players are Sertoli cells, which are connected with germ cells via specialized cell junctions and also form the blood-testis barrier (Lui and Cheng, 2012). In this way, they provide environment for male germ cell development and control all stages of spermatogenesis.

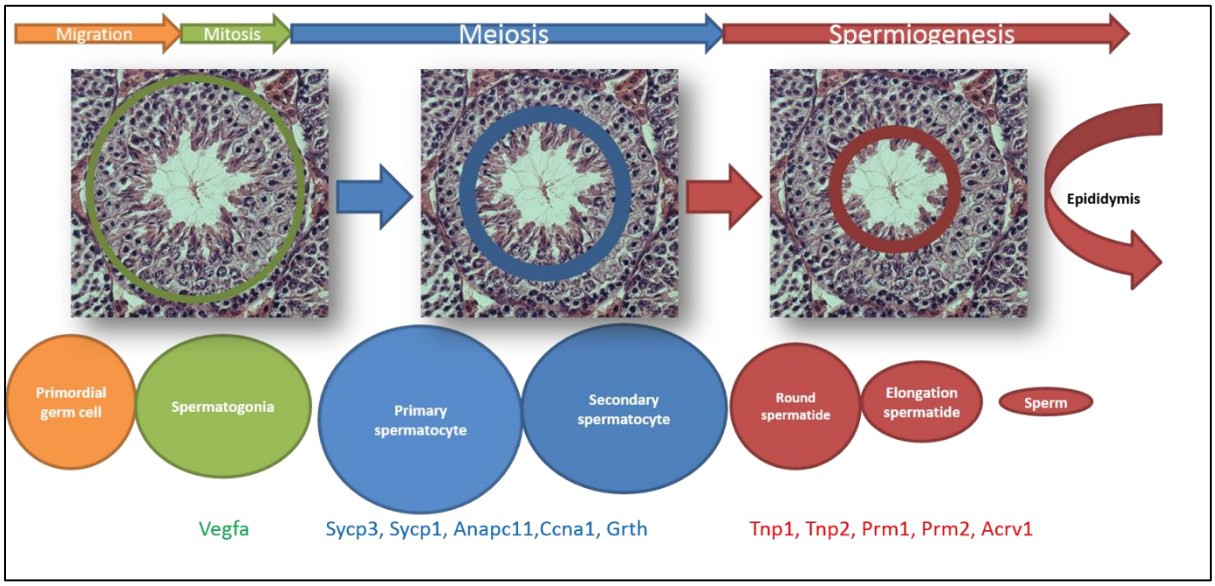

Fig. 3. *Scheme of individual stages of the spermatogenic process with appropriate cell types and selected gene markers*

Extrinsic regulation is represented by the endocrine hormonal system. The main hormones that control development of male germ cells are gonadotropins – luteinizing hormone (LH) and follicle-stimulating hormone (FSH); and sex steroids (e.g., testosterone and 17β-estradiol) (Lui and Cheng, 2012). The hormonal regulation of spermatogenesis is based on the hypothalamo-pituitary-testis axis (Fig. 4). Hypothalamic gonadotrophin-releasing hormone (GnRH) induces secretion of pituitary hormones – LH and FSH. LH acts on Leydig cells, where it induces testosterone production. Testosterone is important for proper spermatogenesis and its absence or absence of functional androgen receptor (AR) cause male infertility. AR is expressed in Leydig cells, peritubular myoid cells and Sertoli cells; however, its presence in germ cells is controversial (Wang et al., 2009). Sertoli cells are considered the main target of the testosterone action. Lack of testosterone or knock out of AR in Sertoli cells results in disruption of the blood-testis barrier and causes

premature release of round spermatids (O'Donnell et al., 1996; Willems et al., 2010). The importance of testosterone action is also illustrated by cyclic expression of AR in Sertoli cells, where certain levels of AR correspond to particular stages of the seminiferous epithelium (Bremner et al., 1994; Suarez-Quian et al., 1999).

Sertoli cells are also target for FSH action. As previously mentioned, in pre-pubertal testes FSH plays a role in determining the number of Sertoli cells (Sharpe et al., 2003). In adult testes, FSH acts as a germ cell surviving factor and promotes the initial stages of spermatogenesis. FSH has been shown to prevent germ cell loss and amplify the population of differentiated spermatogonia in the hypophysectomised Rhesus monkey (Marshall et al., 1995). In Sertoli cells, FSH binding induces expression of genes important for proper spermatogenesis (e.g. follistatin or activin B) and for maintenance of the cyclic character of spermatogenesis (inhibin). Both inhibin and testosterone act via negative feedback on hypothalamo-pituitary axis and inhibit production of LH and FSH (Sofikitis et al., 2008).

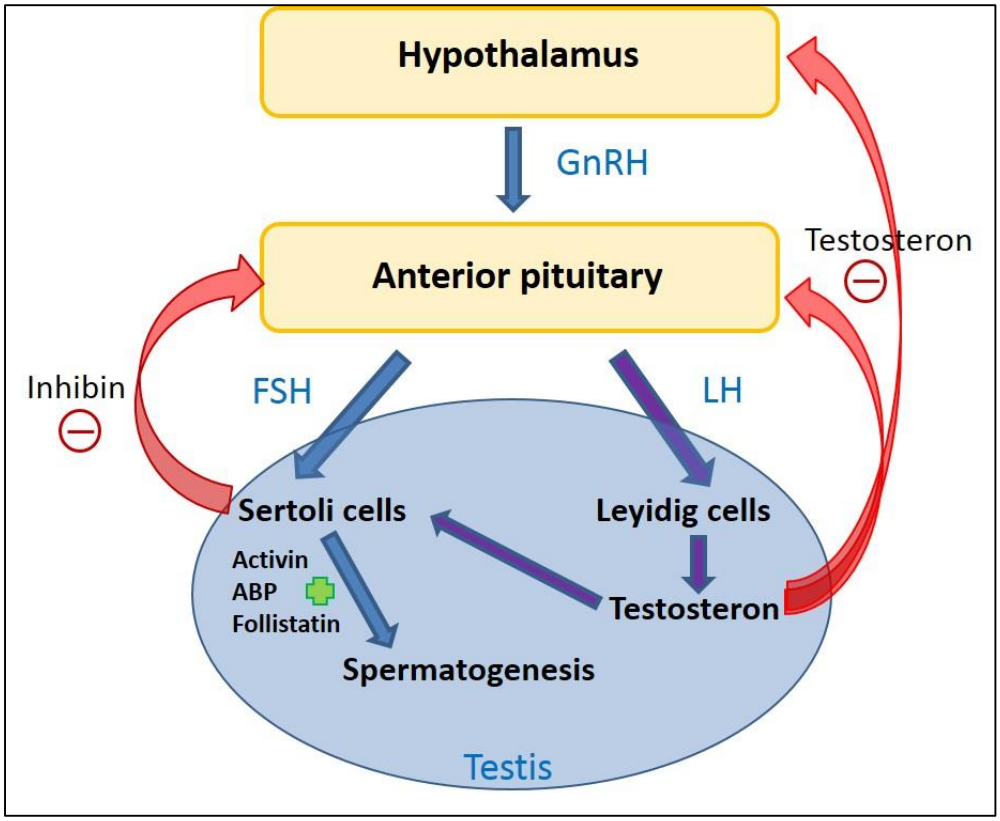

***Fig. 4**. Scheme of hormonal control triggered by hypothalamo-pituitary-testis axis*

In mouse, the entire developmental process from spermatogonial stem cells to spermatozoa takes approximately 35 days (depending on the particular strain). The mitotic stage lasts ~11 days, meiosis lasts ~10 days, and spermiogenesis takes up another ~13.5 days. In humans, the sperm development is nearly twice as long (Eddy, 2002).

1.2. Endocrine disruptors

Endocrine disruptors (EDs) are exogenous substances that are structurally similar to endogenous hormones and are able to interfere with them. This results in disruption of the function of endocrine system, and consequently causes various health problems for the particular organism or its progeny (Damstra et al., 2002). In the last thirty years, these substances are under systematic investigation to reveal their potential harmful effect on mammalian organism. The reproductive system may primarily affect substances with anti-androgenic or estrogenic effect (Crisp et al., 1998).

Anti-androgenic compounds bind to the androgen receptor (AR) and thus prevent natural action of endogenous androgens. The proper function of androgens is most important during the development of the male reproductive tract, when the proper function of androgens is necessary for embryo masculinization. In adults, androgens are important for proper spermatogenesis (Walker, 2011; Smith and Walker, 2014). One example of endocrine disruptor with anti-androgenic action can be vinclozolin, which is widely used as a fungicide (Anway and Skinner, 2008; Elzeinova et al., 2008).

The environmental pollutants with estrogenic action can be divided into phytoestrogens, which are products of plants, and xenoestrogens, which are products of human action. These compounds mimic natural estrogens and can alter the function of estrogen signaling and cause various defects by interfering with synthesis, binding or cellular responses of natural estrogens (Roy et al., 2009). Phytoestrogens include compounds such as genistein (present in soya beans); resveratrol (present in red wine) but also some mycotoxins such as zaeralenone (present in grain) (Fremont, 2000; Kyselova et al., 2004; Cano-Sancho et al., 2013). Xenoestrogens also represent a wide range of compounds. Among the most studied are polychlorinated biphenyls, bisphenol A, phthalates, or diethylstilbestrol (Kuiper-

Goodman et al., 1987; Peknicova et al., 2002; Kyselova et al., 2004; Wittassek and Angerer, 2008).

In the environment, there are many of potential endocrine disruptors which need to be tested for their possible harmful effect (in toxicological studies). The mouse model is widely used in such toxicological studies because of its indisputable advantages. One of them is the existence of various mouse strains with different properties. For example, there are many inbred or outbred mouse strains. Outbred strains are genetically heterogeneous, and thus reflect genetic variability in real human or wild-life population. On the contrary, inbred strains are genetically homogeneous and thus better suited for genetic analysis, and the results are easier to reproduce because there is less genetic variance (Jamsai and O'Bryan, 2011). Another benefit of using a mouse model for reproductive-toxicological studies is their short generation time, which enables performing multigenerational studies. In these studies, mice are exposed to the tested substance for several generations, which allows determination of a possible transgenerational effect of the tested compound.

1.2.1 Tetrabromobisphenol A

Tetrabromobisphenol A (TBBPA) is a highly lipophilic halogen compound, which is commonly used as a flame retardant. With consumption more than 210,000 tons per year, TBBPA is one of the most widely used brominated flame retardants (BFRs) (Alaee et al., 2003). It is primarily used as a reactive flame retardant in epoxy resins and polycarbonates and it has been shown to be able to release from various electronic devices (Birnbaum and Staskal, 2004). Indeed, TBBPA was detected in the environment in various areas. The most frequent sources of emissions are waste water from factories producing BFRs. Samples from a river located near such a factory, revealed quite high concentrations of TBBPA – upstream from the factory – 34 mg of TBBPA/g dry weight; downstream from the factory – 270 mg of TBBPA/g dry weight (Kierkegaard et al., 2009). TBBPA was also found in rivers and marine sediments in Japan (Watanabe et al., 1983) and in the sewage sludge in Sweden and Canada (Kierkegaard et al., 2009) (Fernandez et al., 2007). Sjödin et al. (Sjodin et al., 2001) measured concentrations of TBBPA in the air from the dismantling hall at the recycling plants, computer repair shops, factories producing circuit boards and offices

equipped with computers. TBBPA was found in all the samples, with the highest concentration in recycling plants (55 pmol/m^3). TBBPA was also found in relatively high concentration (19 mg/g) in the dust inside the television cabinets (Takigami et al., 2008) and in the dust of houses in Japan (~500 ng/g) (Takigami et al., 2009). It is very likely that dust is the main source of TBBPA exposure in humans. It was shown that TBBPA may be transported through the placenta to the fetus and it was found in human umbilical cord blood (Legler, 2008).

TBBPA can strongly bind to transthyretin (TTR). The function of TTR is to transport thyroxine (T4) and vitamin A. *In vitro* experiments showed that TBBPA has even higher affinity to TTR than T4 itself (Meerts et al., 2000). Furthermore, it was demonstrated that TBBPA inhibits binding of triiodothyronine (T3) to the thyroid receptor (TR) and stimulates proliferation of GH3 cells (TH-dependent pituitary cells) (Kitamura et al., 2002; Fini et al., 2007). TBBPA is also able to bind to the estrogen receptor *in vitro* (Korner et al., 1998) and is able to induce proliferation of estrogen-dependent MCF-7 (Samuelsen et al., 2001) and Mit/E2 cells (Kitamura et al., 2002). Other *in vitro* studies have shown toxicity of TBBPA to Sertoli TM4 cells. It has been shown that TBBPA increases Ca^{2+} concentration within these cells in the 5–60 µM concentration range. In higher concentrations (18–60 µM), TBBPA also causes cell death via apoptosis (Ogunbayo et al., 2008). A few studies have investigated the effect of TBBPA on the reproductive system *in vivo*. Experiments performed on Wistar rats (Van der Ven et al., 2008) showed reduction of thyroxin circulation, increased weight of gonads and increased plasma levels of testosterone in F1 males.

All these studies indicate that TBBPA is a ubiquitous environmental pollutant with potential negative effect on human or wildlife health. Therefore, additional *in vivo* studies are needed to further study this compound and asses its potential deleterious effect.

1.2.2. Zearalenone

Zearalenone (ZEA) is a mycotoxin produced by fungi of the genus *Fusarium*. These fungi are common pathogens of most agricultural crops mainly wheat, corn, barley, oats, rice, but also hay or silage (Kuiper-Goodman et al., 1987). In mammals, ZEA is metabolized to α- and β-zearalenol (α-ZOL, β-ZOL). Zearalenone and its derivatives are able to bind to

estrogen receptors (ER-α and ER-β) and induce an estrogenic response. ZEA affinity to ERs is about 20 times lower than that of 17β-estradiol (Kuiper-Goodman et al., 1987). ZEA is a thermally stable compound and therefore it is present in various food products, e.g. bread, cereals or milk (Kuiper-Goodman et al., 1987; Prelusky et al., 1990). ZEA concentration in foods varies in a very wide range which depends on climatic conditions (Minervini et al., 2005). Considering the average concentration of ZEA in food and its consumption, the average daily dose was calculated to 2.4 - 29 ng/kg bw/day for an adult in Europe and North America. For toddlers (12–36 months old), the highest average daily intakes was calculated to 9.3 - 100 ng ZEA/kg bw/day (Zinedine et al., 2007). Experiments with radioactively labelled ZEA have shown that ZEA has the same body distribution as estrogen – it was found in the uterus, testicular interstitial cells and ovarian follicles. Some labelled ZEA was also found in adipose tissue where it could accumulate similar way as estrogen (Kuiper-Goodman et al., 1987).

The main effect of zearalenone results from its estrogenic activity. ZEA as well as its derivatives α-ZOL and β-ZOL are able to bind to ER and thus compete with natural 17β-estradiol. It has been shown that binding of ZEA or its derivatives trigger estrogen stimulation (Kuiper-Goodman et al., 1987). Thus ZEA is able to disrupt the hormonal balance in various animals *in vivo*. Breeding animals are often exposed to ZEA because it is commonly present in feed. Different species exhibit distinct sensitivity to ZEA and also relative dose, and life stage during which it is consumed are important. The biggest effect was observed in pigs, where ZEA arouse hyperestrogenism which can lead to vulva swelling, enlargement of the uterus, false pregnancy or abortion (Ruhr et al., 1983). It has been shown that ZEA and its derivatives can also affect the oocytes and sperm *in vitro*. Maturation of pig oocytes i*n vitro* was negatively influenced by α- and β-ZOL (Alm et al., 2002). Negative dose-dependent effect of ZEA was also observed on boar sperm where it induced decrease viability, motility as well as chromatin stability of exposed cells (Tsakmakidis et al., 2006; Benzoni et al., 2008).

Adverse effect of ZEA and its derivate was observed also on mice where it induced significant increase of abnormal spermatozoa and significant decrease of live spermatozoa. Testicular and cauda epididymal sperm counts were also reduced, as well as serum testosterone (Yang et al., 2007a). An *in vivo* study in rats showed that a single

intraperitoneal dose of ZEA (5 mg/kg bw) induces testicular germ cell apoptosis in a time-dependent and stage-specific manner (Kim et al., 2003). In another study, rats were subcutaneously injected with 4 or 40 μg of ZEA at 5^{th}-15^{th} postnatal day and subsequently subjected to analysis at postnatal day 16. This study has shown that treatment with ZEA results in reduced number of spermatogonia and Sertoli cells. Also the testis weight and seminiferous tubule diameter and length were significantly decreased after ZEA exposure (Filipiak et al., 2009). An *in vitro* study, made on mouse Leydig cells, has demonstrated decrease of testosterone production in Leydig cells co-treated with ZEA or α-ZOL and human chorionic gonadotropin. During this study authors also observed decreased expression of 3β-hydroxysteroid dehydrogenase/isomerase, cytochrome P450 side chain cleavage enzyme and steroidogenic acute regulatory protein, which play a crucial role during steroidogenesis (Yang et al., 2007b).

All these results indicate that exposure to ZEA can cause disruption of endogenous hormonal system as well as impair male reproductive parameters *in vivo*. Although, the effect of ZEA was quite extensively investigated, there are no studies examining the long-term exposure to low (physiologically relevant) concentration of ZEA.

1.3. Assisted reproductive technology

As mentioned previously, in human population there are many couples experiencing some form of infertility problems. Many of them search for medical assistance and are recommended to use services of some of the centers of assisted reproduction. In these centers, various assisted reproductive techniques are used. It is very important to choose a suitable technique depending on the cause of infertility. The most simple and least demanding for the couple is intrauterine insemination. In the case of more severe pathology, it is possible to proceed from insemination to conventional *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), testicular sperm extraction (TESE), or microscopic epididymal sperm aspiration (MESA) (Wosnitzer and Goldstein, 2014).

The **conventional IVF** represents a technique in which the oocytes (extracted from the follicles) are placed in a special solution and sperm with normal motility and morphology are subsequently added. The fertilized oocytes are cultivated up to the blastocyst stage

and are then transferred into the uterus. **ICSI** is used in cases of a low number of sperm (oligozoospermia) and motility defects. This methodology is based on incorporation of a single sperm into the oocyte using a micro-needle. Even azoospermia (no measurable level of sperm in the semen) caused by obstruction of the post-testicular genital tracts can be treated. The sperm can be obtained directly from the testes/epididymis with the **TESE**/**MESA** methodology (Mansour, 1998).

The first IVF baby was born in 1978, and it is estimated that since then more than 5 million babies have been born using assisted reproductive technology. Every year, approximately 1.5 million IVF treatments are performed worldwide with about 350,000 babies born. In countries such as Belgium, Czech Republic, Denmark, Estonia, Iceland, Norway, Slovenia and Sweden, more than 3.0% of all babies born were conceived using some of the assisted reproductive techniques (Kupka et al., 2014). These numbers clearly show that assisted reproduction is an important part of our society and there is great effort to further improve the IVF techniques as well as diagnostic methods which are crucial for choosing the most suitable treatment.

2. Aims of the work

- To evaluate how TBBPA affects the male gonadal function with focus on testicular morphology, sperm quality and expression of selected genes;

- To assess the effect of treatment with a low dose of mycotoxine zearalenone on the male gonadal function, sperm quality and expression of genes playing important roles during spermatogenesis, genes expressed in Sertoli cells, and genes playing a role in apoptosis and hormonal response;

- To investigate the expression of spermatogenesis-related genes in specimens from testicular biopsies of infertile men who underwent TESE for the ICSI procedure to provide an additional approach to increase the prediction of positive TESE outcome.

3. Research papers

3.1.

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

Žatecká E., Děd L., Elzeinova F., Kubátová A., Dorosh A., Margaryan H., Dostálová P. and Pěknicová J.

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1. Animals and treatment

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

2.2. Anogenital distance, body and organ weight

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

2.3. Preparation of cells

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

2.4. Sperm morphology and viability

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

2.5. Indirect immunofluorescence

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

2.6. Histological analysis and tissue morphometry

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

2.7. TUNEL analysis

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

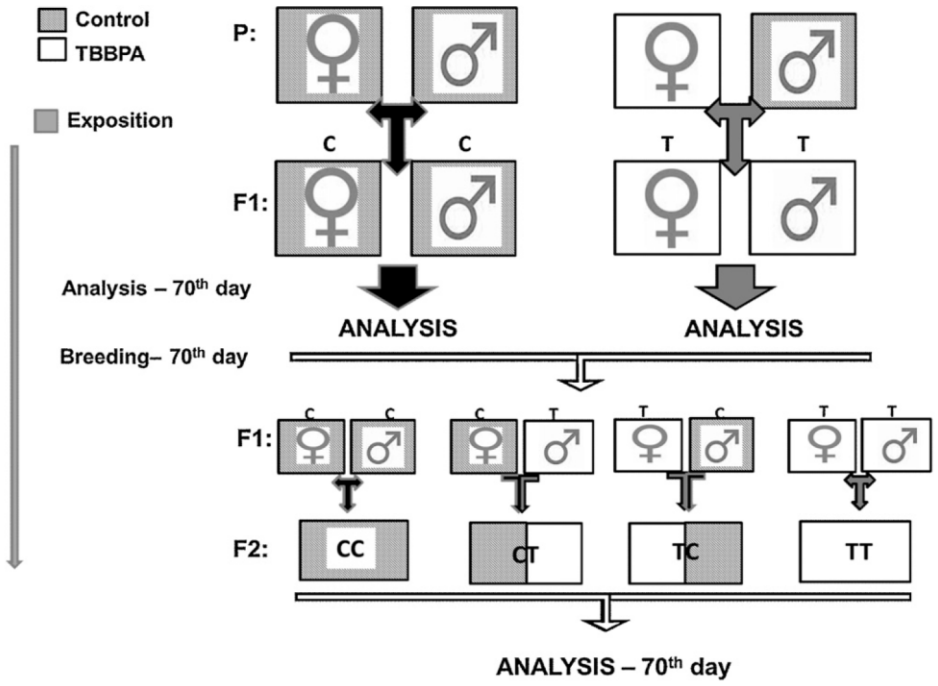

Fig. 1. Breeding diagram. Animals in parental generation were bred to form the F1 generation consisting of two groups (**C**; **T**). In group **C**, born pups were not affected by TBBPA. In group **T**, the pups were exposed to TBBPA in utero and postnatally. Animals of the F1 generation were bred to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

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

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

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

2.9. Statistical analysis

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

3. Results

3.1. Number of progeny; body and organ weight

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

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

Table 1
Primer sequences.

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

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

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

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

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

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

Mean ± SD.

3.2. Sperm parameters

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

3.3. Histology of testes

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

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

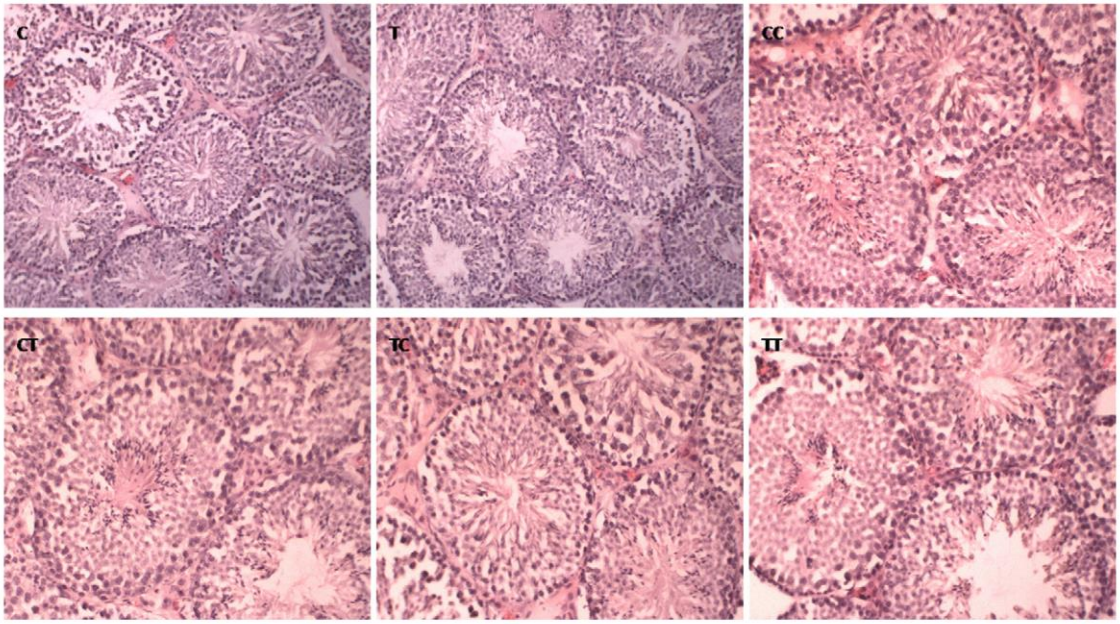

Fig. 2. Histological analysis of the testes from control and experimental animals. Paraffin sections were placed onto glass slides and stained by eosin (cytoplasm) and haematoxylin Harris (nucleus). Histological samples were analyzed under the microscope. Normal morphology and the process of spermatogenesis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

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

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

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

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

3.4. TUNEL analysis

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

3.5. Real time PCR of testicular genes

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

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

5. Conclusion

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

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References

[1] Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environmental Health Perspectives 1993;101(5):378–84.
[2] Elzeinova F, Novakova V, Buckiova D, Kubatova A, Peknicova J. Effect of low dose of vinclozoline on reproductive tract development and sperm parameters in CD1 outbred mice. Reproductive Toxicology 2008;26(3–4):231–8.
[3] Al-Hiyasat AS, Darmani H, Elbetieha AM. Effects of bisphenol A on adult male mouse fertility. European Journal of Oral Sciences 2002;110(2):163–7.
[4] Kyselova V, Peknicova J, Boubelik M, Buckiova D. Body and organ weight, sperm acrosomal status and reproduction after genistein and diethylstilbestrol treatment of CD1 mice in a multigenerational study. Theriogenology 2004;61(7–8):1307–25.
[5] Sharpe RM. Hormones and testis development and the possible adverse effects of environmental chemicals. Toxicology Letters 2001;120(1–3):221–32.
[6] Birnbaum LS, Staskal DF. Brominated flame retardants: cause for concern? Environmental Health Perspectives 2004;112(1):9–17.
[7] Alaee M, Arias P, Sjodin A, Bergman A. An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. Environment International 2003;29(6):683–9.
[8] de Wit CA. An overview of brominated flame retardants in the environment. Chemosphere 2002;46(5):583–624.
[9] Segev O, Kushmaro A, Brenner A. Environmental impact of flame retardants (persistence and biodegradability). International Journal of Environmental Research Public Health 2009;6(2):478–91.
[10] Law RJ, Alaee M, Allchin CR, Boon JP, Lebeuf M, Lepom P, et al. Levels and trends of polybrominated diphenylethers and other brominated flame retardants in wildlife. Environment International 2003;29(6):757–70.
[11] Law RJ, Herzke D, Harrad S, Morris S, Bersuder P, Allchin CR. Levels and trends of HBCD and BDEs in the European and Asian environments, with some information for other BFRs. Chemosphere 2008;73(2):223–41.
[12] de Boer J, Wester PG, van der Horst A, Leonards PE. Polybrominated diphenyl ethers in influents, suspended particulate matter, sediments, sewage treatment plant and effluents and biota from the Netherlands. Environmental Pollution 2003;122(1):63–74.
[13] Darnerud PO. Toxic effects of brominated flame retardants in man and in wildlife. Environment International 2003;29(6):841–53.
[14] Sjodin A, Patterson Jr DG, Bergman A. A review on human exposure to brominated flame retardants—particularly polybrominated diphenyl ethers. Environment International 2003;29(6):829–39.
[15] Watanabe I, Sakai S. Environmental release and behavior of brominated flame retardants. Environment International 2003;29(6):665–82.
[16] Sellstrom U, Jansson B. Analysis of tetrabromobisphenol A in a product and environmental samples. Chemosphere 1995;31:3085–92.
[17] Watanabe I, Kashimoto T, Tatsukawa R. Identification of the flame retardant tetrabromobisphenol-A in the river sediment and the mussel collected in Osaka. Bulletin of Environment Contamination and Toxicology 1983;31(1):48–52.
[18] Lee HB, Peart TE. Organic contaminants in Canadian municipal sewage sludge. Part I. Toxic or endocrine-disrupting phenolic compounds. Water Quality Research Journal of Canada 2002;37(4):681–96.
[19] Sjodin A, Carlsson H, Thuresson K, Sjolin S, Bergman A, Ostman C. Flame retardants in indoor air at an electronics recycling plant and at other work environments. Environmental Science and Technology 2001;35(3):448–54.
[20] Takigami H, Suzuki G, Hirai Y, Sakai S. Brominated flame retardants and other polyhalogenated compounds in indoor air and dust from two houses in Japan. Chemosphere 2009;76(2):270–7.
[21] Takigami H, Suzuki G, Hirai Y, Sakai S. Transfer of brominated flame retardants from components into dust inside television cabinets. Chemosphere 2008;73(2):161–9.
[22] Jakobsson K, Thuresson K, Rylander L, Sjodin A, Hagmar L, Bergman A. Exposure to polybrominated diphenyl ethers and tetrabromobisphenol A among computer technicians. Chemosphere 2002;46(5):709–16.
[23] Kawashiro Y, Fukata H, Omori-Inoue M, Kubonoya K, Jotaki T, Takigami H, et al. Perinatal exposure to brominated flame retardants and polychlorinated biphenyls in Japan. Endocrine Journal 2008;55(6):1071–84.
[24] Meerts IA, van Zanden JJ, Luijks EA, van Leeuwen-Bol I, Marsh G, Jakobsson E, et al. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. Toxicological Sciences 2000;56(1):95–104.
[25] Kitamura S, Jinno N, Ohta S, Kuroki H, Fujimoto N. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. Biochemical and Biophysical Research Communications 2002;293(1):554–9.
[26] Fini JB, Le Mevel S, Turque N, Palmier K, Zalko D, Cravedi JP, et al. An in vivo multiwell-based fluorescent screen for monitoring vertebrate thyroid hormone disruption. Environmental Science and Technology 2007;41(16):5908–14.
[27] Kitamura S, Kato T, Iida M, Jinno N, Suzuki T, Ohta S, et al. Anti-thyroid hormonal activity of tetrabromobisphenol A, a flame retardant, and related compounds: Affinity to the mammalian thyroid hormone receptor, and effect on tadpole metamorphosis. Life Sciences 2005;76(14):1589–601.
[28] Korner W, Hanf V, Schuller W, Bartsch H, Zwirner M, Hagenmaier H. Validation and application of a rapid in vitro assay for assessing the estrogenic potency of halogenated phenolic chemicals. Chemosphere 1998;37(9–12):2395–407.
[29] Samuelsen M, Olsen C, Holme JA, Meussen-Elholm E, Bergmann A, Hongslo JK. Estrogen-like properties of brominated analogs of bisphenol A in the MCF-7 human breast cancer cell line. Cell Biology and Toxicology 2001;17(3):139–51.
[30] Uhnakova B, Ludwig R, Peknicova J, Homolka L, Lisa L, Sulc M, et al. Biodegradation of tetrabromobisphenol A by oxidases in basidiomycetous fungi and estrogenic activity of the biotransformation products. Bioresource Technology 2011;102(20):9409–15.
[31] Van der Ven LT, Van de Kuil T, Verhoef A, Verwer CM, Lilienthal H, Leonards PE, et al. Endocrine effects of tetrabromobisphenol-A (TBBPA) in Wistar rats as tested in a one-generation reproduction study and a subacute toxicity study. Toxicology 2008;245(1–2):76–89.
[32] Peknicova J, Kyselova V, Buckiova D, Boubelik M. Effect of an endocrine disruptor on mammalian fertility. Application of monoclonal antibodies against sperm proteins as markers for testing sperm damage. American Journal of Reproductive Immunology 2002;47(5):311–8.
[33] Peknicova J, Chladek D, Hozak P. Monoclonal antibodies against sperm intra-acrosomal antigens as markers for male infertility diagnostics and estimation of spermatogenesis. American Journal of Reproductive Immunology 2005;53(1):42–9.
[34] Ogunbayo OA, Lai PF, Connolly TJ, Michelangeli F. Tetrabromobisphenol A (TBBPA), induces cell death in TM4 Sertoli cells by modulating Ca^{2+} transport proteins and causing dysregulation of Ca^{2+} homeostasis. Toxicology In Vitro 2008;22(4):943–52.
[35] Sinha Hikim AP, Swerdloff RS. Hormonal and genetic control of germ cell apoptosis in the testis. Reviews of Reproduction 1999;4(1):38–47.
[36] Meinhardt A, Parvinen M, Bacher M, Aumuller G, Hakovirta H, Yagi A, et al. Expression of mitochondrial heat shock protein 60 in distinct cell types and defined stages of rat seminiferous epithelium. Biology of Reproduction 1995;52(4):798–807.
[37] Werner A, Meinhardt A, Seitz J, Bergmann M. Distribution of heat-shock protein 60 immunoreactivity in testes of infertile men. Cell and Tissue Research 1997;288(3):539–44.
[38] Hom JR, Gewandter JS, Michael L, Sheu SS, Yoon Y. Thapsigargin induces biphasic fragmentation of mitochondria through calcium-mediated mitochondrial fission and apoptosis. Journal of Cellular Physiology 2007;212(2):498–508.
[39] Feng HL, Sandlow JI, Sparks AE. Decreased expression of the heat shock protein hsp70-2 is associated with the pathogenesis of male infertility. Fertility and Sterility 2001;76(6):1136–9.
[40] Dix DJ, Allen JW, Collins BW, Poorman-Allen P, Mori C, Blizard DR, et al. HSP70-2 is required for desynapsis of synaptonemal complexes during meiotic prophase in juvenile and adult mouse spermatocytes. Development 1997;124(22):4595–603.
[41] Widlak W, Vydra N, Malusecka E, Dudaladava V, Winiarski B, Scieglinska D, et al. Heat shock transcription factor 1 down-regulates spermatocyte-specific 70 kDa heat shock protein expression prior to the induction of apoptosis in mouse testes. Genes to Cells 2007;12(4):487–99.
[42] Baler R, Dahl G, Voellmy R. Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. Molecular and Cellular Biology 1993;13(4):2486–96.
[43] Eddy EM. Role of heat shock protein HSP70-2 in spermatogenesis. Reviews of Reproduction 1999;4(1):23–30.
[44] Eddy EM. Male germ cell gene expression. Recent Progress in Hormone Research 2002;57:103–28.
[45] Peknicova J, Capkova J, Geussova G, Ivanova M, Mollova M. Monoclonal antibodies to intra-acrosomal proteins inhibit gamete binding in vitro. Theriogenology 2001;56(2):211–23.
[46] Ohsako S, Kubota K, Kurosawa S, Takeda K, Qing W, Ishimura R, et al. Alterations of gene expression in adult male rat testis and pituitary shortly after subacute administration of the antiandrogen flutamide. Journal of Reproductive Development 2003;49(4):275–90.
[47] Stouder Ch, Paolini-Giacobino A. Transgenerational effect of the endocrine disruptor vinclozoline on the methylation pattern of imprinted genes in the mouse sperm. Reproduction 2010;139:373–9.
[48] Marques CJ, Francisco T, Sousa S, Carvalho F, Barros A, Sousa M. Methylation defect of imprinted genes in human testicular spermatozoa. Fertility and Sterility 2010;94(2):585–94.
[49] Minor A, Chow V, Ma S. Aberrant DNA methylation at imprinted genes in testicular sperm retrieved from men with obstructive azoospermia and undergoing vasectomy reversal. Reproduction 2011;141(6):749–57.
[50] Kobayashi H, Sato A, Otsu E, Hiura H, Tomatsu C, Utsonomiya T, et al. Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. Human Molecular Genetics 2007;16(21):2542–51.
[51] Marques CJ, Costa P, Vaz B, Carvalho F, Fernandes S, Barros A, et al. Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. Molecular Human Reproduction 2008;14(2):67–74.

3.2.

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The effect of tetrabromobisphenol A on protamine content and DNA integrity in mouse spermatozoa

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SUMMARY

Tetrabromobisphenol A (TBBPA) is a widely used brominated flame retardant of increasing concern to human health because of its action as an endocrine disruptor. We have previously demonstrated that TBBPA is able to increase apoptosis of testicular cells and other changes in the first and second generations of mice exposed to TBBPA. However, the potential effects of TBBPA on mouse epididymal spermatozoa have not yet been investigated. Therefore, we initiated this study to determine whether TBBPA exposure could also result in increased DNA fragmentation in epididymal spermatozoa and whether it had an effect on the protamines as the major nuclear proteins. C57Bl/6J mouse pups (n = 10) were exposed to TBBPA (experimental group) during the gestation, lactation, pre-pubertal and pubertal periods up to the age of 70 days as previously described and compared to control mouse pups (n = 10) that were not exposed. The results demonstrate that TBBPA treatment results in a significantly decreased protamine 1/protamine 2 ratio (0.362 vs. 0.494; $p < 0.001$), increased total protamine/DNA ratio (0.517 vs. 0.324; $p < 0.001$) and increased number of terminal deoxynucleotidyl transferase dUTP nick end labelling positive spermatozoa (39.5% vs. 21.2%; $p < 0.05$) observed between TBBPA and control mice respectively. These findings indicate that TBBPA exposure, in addition to the resulting increased sperm DNA damage, also has the potential to alter the epigenetic marking of sperm chromatin through generation of an anomalous content and distribution of protamines. The possibility is now open to study whether the detected altered protamine content and DNA integrity are related to the previously observed second-generation effects upon TBBPA exposure.

INTRODUCTION

Tetrabromobisphenol A (TBBPA) is a brominated flame retardant with a global consumption of 210 000 tonnes per year (European Food Safety Authority, 2011) and is released into the environment from existing or wasted products exposing the human population. For example, TBBPA was found in the blood serum of computer technicians, in breast milk and blood serum of mothers, and has also been shown to be transported through the placenta to the foetus (Jakobsson *et al.*, 2002; de Wit, 2002; Cariou *et al.*, 2008; Kawashiro *et al.*, 2008). Brominated flame retardants are classified as endocrine disruptors because they mimic the function of endogenous hormones (Korner *et al.*, 1998; Meerts *et al.*, 2000; Samuelsen *et al.*, 2001; Kitamura *et al.*, 2002; Uhnakova *et al.*, 2011). Few studies have examined the effect of TBBPA on the reproductive system in vivo. Experiments performed on Wistar rats (Van der Ven *et al.*, 2008) reported a reduction of thyroxin in circulation and an increase in gonad weight and plasma levels of testosterone in F1 males. A previous study conducted in our laboratory tested the effect of TBBPA on reproductive parameters in a two-generational in vivo study using CD1 mice. We observed increased apoptosis of testicular cells and changes in the expression of genes playing important roles during spermatogenesis. A parental effect related to TBBPA in the second generation was also found in this study (Zatecka *et al.*, 2013).

This study was designed to examine the effect of TBBPA on spermatozoa as the carriers of parental genetic information. The spermatozoon is a specialized cell with a condensed nucleus, in which the majority of the DNA is tightly packaged in toroidal structures by protamines (Oliva & Dixon, 1991; Oliva, 2006; Balhorn, 2007; Carrell *et al.*, 2008; Oliva & Castillo 2011). In mice as well as humans, there are two types of protamines: protamine 1 (P1) and protamine 2 (P2). Several studies have reported an altered amount of protamines in the sperm cell of infertile patients and a correlation with DNA fragmentation (Corzett *et al.*, 2002; Oliva, 2006; Balhorn, 2007; Carrell *et al.*, 2008; de Mateo *et al.*, 2009). Furthermore, haploinsufficiency of only one of the protamine 1 gene (*Prm1*) or protamine 2 gene (*Prm2*)

alleles in knockout mice models results in severely altered spermatogenesis, increased DNA damage and sperm cell apoptosis (Cho *et al.*, 2001, 2003). Indeed, one of the main functions of sperm protamines is related to the protection of the paternal genetic message through making it inaccessible to nucleases or mutagens (Oliva, 2006). This is especially relevant as sperm genome integrity seems to be necessary to accomplish fertilization (Tomsu *et al.*, 2002; Simon *et al.*, 2011). However, protamines may also be involved in paternal genome imprinting during spermatogenesis and in the constitution of epigenetic marks potentially transmitted to the oocyte upon fertilization (Oliva & Dixon, 1991; Oliva, 2006; Carrell *et al.*, 2008; Hammoud *et al.*, 2011; Castillo *et al.*, 2014). Despite all the above studies correlating TBBPA with male reproductive effects and the links between altered protamination, DNA fragmentation and male infertility, to our knowledge, there are no studies measuring the effect of TBBPA on protamination of the sperm cell and DNA integrity. Therefore, this study was performed to determine the effect of TBBPA on protamination levels and sperm DNA integrity as both parameters are correlated with male fertility.

MATERIALS AND METHODS

Animals and treatment

For our experiment, we used a C57Bl/6J inbred mouse strain (An Lab, Prague, Czech Republic). Mice were kept under standard experimental conditions with constant temperature (23–24 °C) and a 12-h light regime in the animal facility of the Institute of Molecular Genetics of the AS CR, v. v. i., Prague. Animals were fed on soy-free feed (LASvendi, Soest, Germany). Food and water were administered ad libitum. In this in vivo experiment, there were two groups; one group exposed to TBBPA (experimental group) and the control group, both exposed in the indicated animal facility in Prague. In each group, 10 animals were analysed. Experimental animals were treated with TBBPA (Sigma, Prague, Czech Republic), which was dissolved in drinking water in a concentration of 200 μg/L. The animals were exposed approximately to 1 μg of TBBPA/mouse per day (equivalent to 35 μg/kg). The concentration of TBBPA in the environment is highly variable, but we tried to select our concentration to mimic potential in vivo exposures (Sellstrom & Jansson, 1995; Takigami *et al.*, 2009), and also because it had been used in our previous in vivo study where we showed that TBBPA is able to influence processes occurring in the testicular tissue (Zatecka *et al.*, 2013).

Animals in the experimental group were exposed from the day of conception (their parents were non-exposed animals) throughout the gestation, lactation, pre-pubertal and pubertal periods up to the age of 70 days, and then they were sacrificed and subjected to analysis. Each group (experimental and control) consisted of 10 individuals. The animals were exposed during all their life since conception in order to simulate real exposures of humans to environmental pollutants throughout life. All procedures were approved by the Committee for Animal Welfare and Protection.

Sperm extraction and purification

The epididymides were dissected and cleaned from any additional tissue. The caudal regions of the epididymides were separated, placed into warmed (37 °C) PBS and then they were carefully cut into small pieces, and the spermatozoa were left to release spontaneously from the epididymis in a CO_2 incubator (CO_2 5%) for 15 min. After incubation, the mixture was poured through a 30-μm filter to obtain only the sperm cell fraction (Partec, Görlitz, Germany) and PBS was added to 1 mL final volume. Part of the epididymal spermatozoa was used for assessing sperm morphology and DNA damage. The rest of the suspension was washed in PBS, centrifuged at 1240 ***g*** for 10 min at 4 °C and the pellet was used for the protamine extraction.

Sperm morphology

For evaluation of the morphological state of spermatozoa, 10 μL of sperm cell suspension was placed onto a glass slide, fixed at 37 °C and labelled by Spermac Stain System according to the manufacturer's protocol (Ferti Pro, Beernem, Belgium). A total of 200 cells from each sample were evaluated as described (Elzeinova *et al.*, 2008).

Testosterone and T3 level measurements

Blood samples (0.5 mL) were collected from males at the age of 70 days. Serum testosterone and total triiodothyronine (T3) were measured by the commercially available RIA kits (Beckman coulter, Prague, Czech Republic) according to manufacturer's instructions. Serum samples were evaluated using a gamma counter (Cobra II; Canberra Packard, Meriden, CT, USA) set for 125 iodine.

Sperm DNA damage

The level of DNA damage of epididymal spermatozoa was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), using a detection kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The fresh sperm suspension (final concentration of 1×10^6 cells/mL) was dropped onto three-well diagnostic microscope slides (ThermoScientific, Portsmouth, NH, USA) and air-dried. For each well, 10 μL of sperm suspension was used. Subsequently, the samples were processed according to manufacturer's instructions. Finally, the samples were mounted in Vectashield with DAPI dye (Vector, Burlingame, CA, USA) for DNA visualization (Domínguez-Fandos *et al.*, 2007). All specimens were evaluated with a fluorescent microscope Nikon Eclipse 400 (Nikon, Tokyo, Japan) equipped with a Nikon Plan Apo VC 60/1.40 oil objective (Nikon). The images were photographed with a CCD camera 1300-VDS (Vosskühler, Osnabrück, Germany) with the aid of the NIS-Elements Ar imaging software (Laboratory Imaging, Prague, Czech Republic). Two hundred cells were evaluated in each sample.

Extraction of sperm nuclear proteins

For protamine extraction, 5×10^6 of mouse epididymal sperm cells were used. Sperm cells were resuspended in 200-μL PBS, centrifuged at 8940 ***g*** for 5 min at 4 °C and the pellet was resuspended in 100 μL of 0.5% Triton X-100, 20 mM Tris, 2 mM $MgCl_2$ solution and centrifuged at 8940 ***g*** for 5 min at 4 °C. After centrifugation the samples were treated as described previously (de Yebra & Oliva, 1993), except that instead of using iodoacetate treatment we performed a treatment with 0.8% vinylpyridine (Sigma) for 30 min at 37 °C to further inhibit formation of cysteine disulphide bonds. Finally, each sample was resuspended in 10 μL of sample buffer containing 5.5 M urea, 20% β-

mercaptoethanol and 5% acetic acid. The DNA remaining in the pellet after the extraction of the nuclear proteins with 0.5 M HCl was extracted and quantified after 0.5 N perchloric acid hydrolysis (90 °C, 20 min) and absorbance determination at 260 nm, measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA), as described previously (Castillo *et al.*, 2011).

Separation and analysis of sperm nuclear proteins

Basic nuclear proteins were analysed using acid–urea polyacrylamide gel containing 2.5 M urea, 12.5 mM thiourea, 0.9 M acetic acid, 15% acrylamide, 0.1% bis-acrylamide and 0.12% H_2O_2. After polymerization, 2 μL of each sample was loaded and the gel was electrophoresed in 0.9 M acetic acid buffer for 90 min at 110 V (Ausio, 1992). Different known quantities of a human protamine standard from a pool of human normozoospermic sperm samples (0.435, 0.87, 1.74 and 2.61 μg) were added in each acid–urea electrophoretic gel (Mengual *et al.*, 2003).

The gels were stained with EzBlue staining reagent (Sigma) following the manufacturer's instructions. The stained gels were scanned and the intensity of the bands corresponding to P1 and P2 was quantified with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). The data obtained from Quantity One software were used for calculating the P1/P2 ratio. A standard curve was obtained from the different concentrations of human protamine standard to calculate the total amount of protamines (P1 + P2) in each sample, and the P1/P2, P1 + P2/DNA, P1/DNA and P2/DNA ratios were calculated (Mengual *et al.*, 2003). A representative example of one of the acid–urea polyacrylamide gels stained by EzBlue is shown in Fig. 1.

Reverse transcription–polymerase chain reaction

The whole RNA was extracted from the whole testicular tissue using the Tri-Reagent kit (Sigma). Briefly, to each testicle, 1 mL of Tri-Reagent was added and the tissue was then homogenized in homogenizer Precellys 24 (Bertin Technologies, Aix-en-Provence, France). Subsequently, the samples were processed according to the manufacturer's instructions. The quality and purity of the isolated RNA was measured with a NanoDrop-1000 spectrophotometer (Thermo Scientific, Washington, DC, USA). The cDNA was synthesized by combining 5 μg of purified RNA, 1 μL DNase I (Invitrogen, Carlsbad, CA, USA), 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, VT, USA). After incubation, 1 μL EDTA (Fermentas) was added and the mixture was incubated at 65 °C for 10 min. The volume of 30 μL of the reaction mixture (8 μL of reaction buffer for M-MuLV reverse transcriptase (Fermentas), 5 μL 10 mM 4 dNTP (Fermentas), 0.3 μL RiboLock inhibitor (Fermentas), 1 μL oligo (dT) + random primers (Promega) and 15.2 μL H_2O) was added to the samples. The mixture was incubated for 60 min at 42 °C and then for 10 min at 70 °C. The obtained cDNA was stored at −20 °C for subsequent analysis.

Quantitative reverse transcription–polymerase chain reaction

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed with primers for *Prm1*, *Prm2*, transition protein 1 gene (*Tnp1*) and transition protein 2 gene (*Tnp2*). The primer sequences are given in Table 1. For each reaction, 2 μL of 5x 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 (Bio-Rad Laboratories). The relative amount of mRNA in each sample was calculated from the measured CT values. The control was set at 100% and the experimental samples were compared to the control. The expression of the gene for β-actin (Actb) was used to optimize the measured values as described (Livak & Schmittgen, 2001).

Statistical analysis

Experimental data were analysed using STATISTICA 6.0 (DELL-StatSoft, Inc, Tulsa, OK, USA) and GraphPad Prism 5.04 (GraphPad Software, Inc., La Jolla, CA, USA). The differences between individual parameters in the control and the TBBPA group were tested by Mann–Whitney *U*-test. The correlations between individual parameters were expressed as Pearson product-moment correlation coefficient (*r*). Individual *r* coefficients were tested for their significance. A *p* value equal or lower than 0.05 was considered significant ($*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$).

RESULTS

General reproductive system measurements and animal body weight

TBBPA treatment did not result in statistically significant changes in any of the general reproductive system parameters measured (Table S1). Recorded parameters were the ano-genital distance, weight of the reproductive organs, sperm morphology and concentration. Also, the animal body weight was not changed as a result of the treatment.

Testosterone and T3 levels

Testosterone and T3 levels were measured to determine whether the previous reported effects of TBBPA treatment in rats (Van der Ven *et al.*, 2008) could also be reproduced in mice. As expected, TBBPA-treated animal had lower testosterone levels as compared to the control group (0.268 ng/mL ± 0.23 (SD) vs. 0.48 ng/mL ± 0.222 (SD); $p < 0.05$). The average T3 levels were also lower in the TBBPA-treated animals, although in this case no significant differences were reached (1.187 nmol/L ± 0.324 (SD) vs. 1.301 nmol/L ± 0.135 (SD).

Protamine ratio and protamine content

After protein staining and visualization, we measured the optical density of the bands that corresponded to P1 and P2 respectively (Fig. 1A), and the P1 to P2 ratio (P1/P2) was calculated for each sample (Fig. 1B). In animals exposed to TBBPA, the P1/P2 ratio was 0.362 ± 0.024, whereas in control animals the ratio was 0.494 ± 0.052 ($p < 0.001$), consistent with the normal ratio described for mice (Corzett *et al.*, 2002). Thus, in the experimental animals, the P1/P2 ratio was significantly lower as compared to the control group (Fig. 1B). Using protamine standards, we also calculated the whole protamine content in each sample. Finally, we also measured the DNA content of the samples, which allowed calculating the total protamine to DNA ratio (P1 + P2/DNA) and the P1/DNA and P2/DNA ratios. In the control animals, the P1 + P2/DNA ratio was 0.324 ± 0.081, whereas in the experimental animals we obtained a significantly higher

Figure 1 Analysis of protamine 1 (P1), protamine 2 (P2) and protamine ratios in control and tetrabromobisphenol A (TBBPA)-treated groups. (A) Representative examples of the visualization of protamines in mouse sperm samples from control and TBBPA-treated animals. Proteins were extracted from epididymal spermatozoa, separated on acid–urea polyacrylamide gel and stained with EzBlue. Some representative examples are shown. (B) P1 to P2 ratio (P1/P2) in epididymal spermatozoa. The Mann–Whitney *U*-test shows that the P1/P2 ratio is significantly lower in animals exposed to TBBPA. (C) Protamine to DNA ratio in epididymal spermatozoa. (D) P1/DNA ratio. (E) P2/DNA ratio. The Mann–Whitney *U*-test shows that the P1 + P2/DNA ratio is significantly higher in animals exposed to TBBPA. ***$p < 0.001$. Mean ± SD; $n = 10$/group.

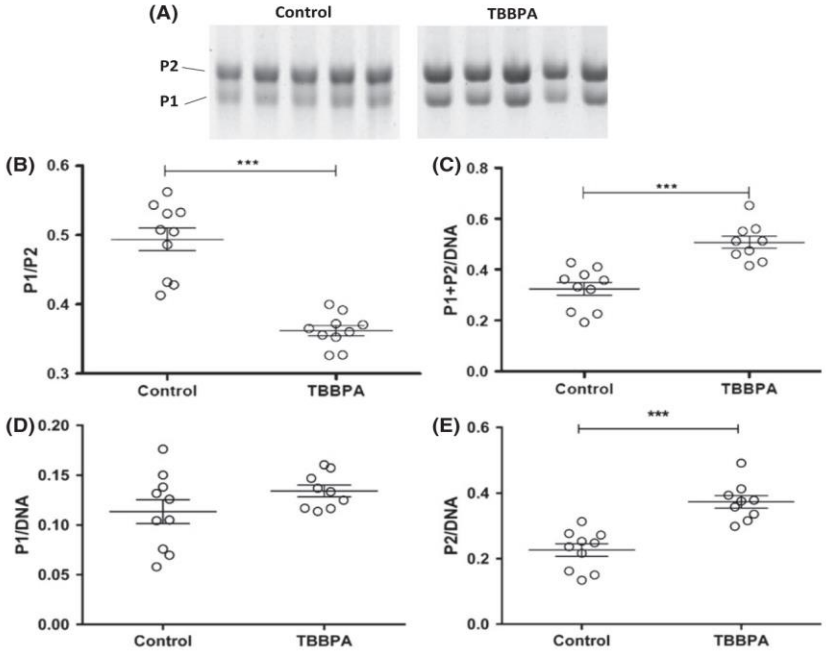


Table 1 Primer sequences used in the RT-PCR experiments

Gene	Accession no.	Nucleotide sequence 5′–3′	Size of PCR product (bp)
Actb	NM_007393.3	CGGTTCCGATGCCCTGAGGCTCTT CGTCACACTTCATGATGGAATTGA	100
Prm1	NM_013637.4	ACAGGTTGGCTGGCTCGACC CGGCAGCATCGGTATCTGGCC	90
Prm2	NM_008933.1	CCAGGGGCCTGGACAAGACC TCTGTGGTGGTGGTGGCCCC	112
Tnp1	NM_009407.2	CCGAGCTCCTCACAAGGGCGT CAGGGCAGAGCTCATTGCCGC	140
Tnp2	NM_013694.4	CCTGCAAGACCCAGCCACCG GTTTCCGCCTCCTGACGGCC	94

RT-PCR, reverse transcription–polymerase chain reaction; Actb, β-actin; *Prm1*, protamine 1; *Prm2*, protamine 2; *Tnp1*, transition protein 1; *Tnp2*, transition protein 2.

P1 + P2/DNA ratio of 0.517 ± 0.073 ($p < 0.001$; Fig. 1C). This increase in the total protamine content is due to a significant increase in the P2/DNA ratio (Fig 1E),

Sperm morphology and DNA damage

The cauda epididymal spermatozoa obtained from control and experimental animals were subjected to morphological analysis and evaluation of DNA damage through the TUNEL assay, which enables the detection of DNA fragmentation. There were no differences in the number of sperm cells/mL recovered (the sperm cells × 10^6/mL ± SD was 17.60 ± 6.40 in control vs. 15.76 ± 5.80 in treated animals). During the morphological analysis, we evaluated the morphology of sperm heads. We did not observe any differences in the number of morphologically abnormal spermatozoa in TBBPA-treated animals compared to the control (% of average abnormal spermatozoa ± SD was 6.10 ± 2.60 vs. 7.6 ± 2.59 respectively). However, as shown in Fig. 2, we detected a significantly higher number of TUNEL-positive cells in spermatozoa from TBBPA-treated animals (39.5 ± 4.5% cells) as compared to the controls (21.2 ± 3.1% cells; $p < 0.05$). Moreover, the statistical analysis revealed a negative correlation between the DNA damage and the P1/P2 ratio (Fig. 3; $p < 0.05$). However, we did not find any correlation between total protamine/DNA; P1/DNA; P2/DNA and sperm DNA damage. In case of P2/DNA with TUNEL we could see a trend ($r = 0.38$); however, the p value did not reach level of

Figure 2 Number of cells positive for terminal deoxynucleotidyl transferase dUTP nick end labelling assay (TUNEL expressed as %). The Mann–Whitney *U*-test shows that spermatozoa of animals treated with tetrabromobisphenol A (TBBPA) display higher DNA damage compared to the control group (*$p < 0.05$. Mean ± SD; $n = 10$/group).

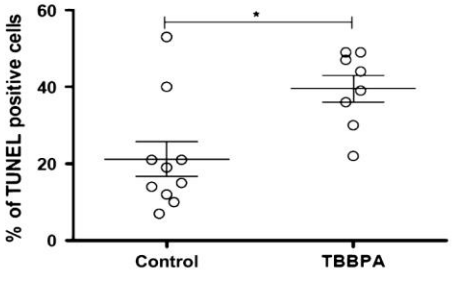
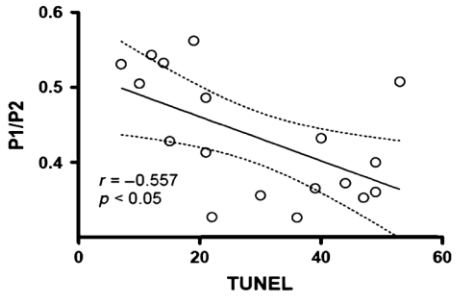


Figure 3 Correlation between DNA damage [number of terminal deoxynucleotidyl transferase dUTP nick end labelling assay (TUNEL) positive cells in %] and protamine 1 (P1)/protamine 2 (P2) ratio. The statistical analysis shows that there is a significant negative correlation between these two parameters (*$p < 0.05$. Mean ± SD; $n = 10$/group).



significance ($p = 0.11$). We also searched for potential correlations between the sperm concentration or morphology and sperm protamination, but we did not find any significant results. We did not detect any correlation either between sperm morphology assessed using optical microscopy and protamine content. However, it will be interesting to explore in the future, using higher resolution methods, whether the detected alterations in protamine content are linked to ultrastructural defects.

Expression analysis of testicular RNA

After testes dissection, we extracted whole testicular RNA and evaluated the expression of several testicular genes. Genetic analysis was performed by qRT-PCR with primers for the *Prm1*; *Prm2*; *Tnp1* and *Tnp2* genes (Table 1). The qRT-PCR method did not reveal any significant changes in the expression profile of any of the tested genes in the experimental animals (Table 2). We also calculated the Prm1/Prm2 mRNA ratio. In experimental animals, the ratio was slightly lower (average 0.56 ± 0.10 vs. 0.60 ± 0.18); however, the differences were not statistically significant.

DISCUSSION

In this manuscript, we report that TBBPA exposure leads to a decreased P1/P2 ratio, increased total protamine/DNA ratio and in an increased number of TUNEL-positive spermatozoa (Figs 1 and 2). In humans, alterations in the P1/P2 ratio have been reported in infertile patients and are associated with increased sperm DNA damage and failed assisted reproduction outcomes (Mengual *et al.*, 2003; Aoki *et al.*, 2005; Balhorn, 2007; de Mateo *et al.*, 2009; Castillo *et al.*, 2011; Simon *et al.*, 2011). According to the DNA protection hypothesis for protamines, the prediction would be that the detected increase in DNA damage would correlate with decreased total P1 + P2/DNA ratios (Aoki *et al.*, 2005; Oliva, 2006). However, our results obtained in the present work in the mouse model instead indicate a significant increase in the total P1 + P2/DNA ratio in the TBBPA-treated animals, with increased TUNEL-positive spermatozoa (Figs 1 and 2). But not all published data fit well the DNA protection hypothesis. For example, a significantly increased P1 + P2/DNA ratio was detected in the sperm cells from native semen infertile patient samples having higher DNA fragmentation levels as compared to density gradient-selected spermatozoa with lower levels of DNA damage and lower P1 + P2/DNA ratios (Castillo *et al.*, 2011). Also, a lack of detection of significant changes in the total protamine content was reported in oligozoospermic patients as compared to controls, despite the presence of significantly different P1/P2 ratios (Mengual *et al.*, 2003). Differently to the above data on protamine content and DNA damage, low P1/P2 ratios have been consistently associated with higher sperm DNA fragmentation indices in different studies (Aoki *et al.*, 2005; Oliva, 2006; Fig. 3). Thus, the P1/P2 and protamine/DNA (or protamine/spermatozoa) ratios are not equivalent. Therefore, our results along with some of these previous observations suggest that the detected associations between decreased P1/P2 ratios, changes in the protamine/DNA ratios found in some studies and increased DNA damage cannot be simply explained by the DNA protection hypothesis of protamines, but that the link is more complex.

Another hypothesis for the function of protamines is that they are involved in the imprinting of sperm chromatin (Oliva & Dixon, 1991; Oliva, 2006; Arpanahi *et al.*, 2009; Hammoud *et al.*, 2009; Castillo *et al.*, 2014). This latter imprinting hypothesis has recently received substantial support, since approximately 1 and 8% of the mouse and human sperm chromatin, respectively, was found not to be organized by protamines, but associated with sperm nucleosomes that are enriched in genes important for the initial embryo developmental stages (Gatewood *et al.*, 1987; Gardiner-Garden *et al.*, 1998; Zalenskaya *et al.*, 2000; Wykes & Krawetz, 2003; Arpanahi *et al.*, 2009; Hammoud *et al.*, 2009). The rest of the different chromatin-packaging genes is organized in nucleoprotamines (approximately 99% in mouse spermatozoa and 92% in human spermatozoa) (Arpanahi *et al.*, 2009; Hammoud *et al.*, 2009). Furthermore, alterations were detected in the gene distribution in nucleoprotamine and nucleohistone domains of infertile patients (Hammoud *et al.*, 2011). Also, histone retention is altered in mouse impaired spermatogenesis (Ihara *et al.*, 2014). Therefore, the reported associations between decreased P1/P2 ratios, increased DNA fragmentation and infertility cannot be simply explained by the lack of protection of the chromatin, but may be the consequence of a complex and general sperm chromatin disordering resulting in altered epigenetic imprinting, decreased P1/P2 ratios, altered protamine/DNA content and increased DNA damage (Carrell *et al.*, 2008; Oliva &

Table 2 Quantitative reverse transcription–polymerase chain reaction analysis of testicular protamine 1 (*Prm1*); protamine 2 (*Prm2*); transition protein 1 (*Tnp1*) and transition protein 2 (*Tnp2*) genes

Group	*Prm1* (%)	*Prm2* (%)	*Tnp1* (%)	*Tnp2* (%)	*Prm1*/*Prm2*
Control	100.0 ± 38.9	100.0 ± 47.2	100.0 ± 26.1	100.0 ± 16.9	0.56 ± 0.10
TBBPA	91.2 ± 28.0	100.1 ± 29.3	100.8 ± 20.5	100.1 ± 16.4	0.60 ± 0.18
p Value	0.76	0.59	0.95	0.99	0.66

Relative expression of the selected genes and the *Prm1*/*Prm2* ratio are given. The control group represents 100% and the percentage in the experimental group represents the ratio of gene expression between the experimental (TBBPA) and the control group. No significant differences were detected. TBBPA, tetrabromobisphenol A.

Ballesca, 2012; Castillo *et al.*, 2014; Jodar & Oliva, 2014). Our present results would be consistent with a general sperm chromatin disordering rather than with the classical DNA protection hypothesis. It would be interesting in future studies to measure the effect of TBBPA on the gene distribution in the nucleoprotamine and nucleohistone-packaged regions in spermatozoa and associated DNA methylation patterns.

Although a high variability of P1/P2 has been detected among mammals, a particular proportion of the two protamines seems to be important within the same species (Corzett *et al.*, 2002). Some mammalian species do not have detectable levels of protamine 2 (such as boar and rat; Oliva & Dixon, 1991; Corzett *et al.*, 2002). There are even many vertebrate species with no protamines at all in their sperm cells (such as zebra fish and goldfish), and yet they efficiently manage to transmit intact paternal genomes to the offspring. For example, the zebrafish is a fish species that, instead of protamines, uses increased amounts of linker histone H1 and depletion of chromatin-decondensing modifications (such as H4K16ac) to accomplish sperm chromatin condensation, and where genes important for embryo development are packaged in blocks of multivalent chromatin (Wu *et al.*, 2011). It is interesting to note that different endocrine disruptors have been reported to result in sperm epimutations and increased TUNEL-positive cells in the testis of the corresponding animals (Guerrero-Bosagna *et al.*, 2012; Manikkam *et al.*, 2013). The present detection of increased sperm DNA fragmentation in the TBBPA-treated group is also consistent with the recently reported effect of TBBPA in the sperm cells of the starlet fish measured using COMET assay (Linhartova *et al.*, 2014). Increased sperm DNA damage has also been reported for men exposed to other endocrine disruptors such as bisphenol A (Rochester, 2013). Of course, the imprinting hypothesis and the DNA protection hypothesis for protamines are not mutually exclusive and could perfectly both coexist and be valid.

We have not detected any correlation between the P1/P2 alterations present in the spermatozoa and the protamine transcript levels in the testis (Table 2). Therefore, the protamine alterations detected at the protein level in our study are not due to a transcriptional alteration in the corresponding genes, but must have originated through post-translational processing and/or deposition onto the chromatin. In humans, alterations in protamine transcripts have been described in the testis (Steger *et al.*, 2000, 2001, 2003) and mature spermatozoa of infertile patients (Jodar *et al.*, 2012). Therefore, the mechanisms involved appear to be different in our model from those operating in infertile patients. Separate analysis of the P1/DNA and P2/DNA ratios in the two groups indicates that the abnormally increased P1 + P2/DNA ratio in the TBBPA-treated animals is mainly due to a significant increase in the P2/DNA ratio (Fig. 1e). The main difference between the two protamines is that P1 is directly translated into the mature form, whereas P2 is first synthesized as a precursor and then proteolytically processed to give rise to the mature form of P2 (Torregrosa *et al.*, 2006; de Mateo *et al.*, 2009, 2011). But the increased ratio detected excludes a defect of processing. A potential explanation could be that the transcripts for P1 and P2 are produced normally, but there is some effect of the TBBPA exposure on Prm2 transcript stabilization or translation that could result in overproduction of P2. The possibility is now open in future studies to further clarify the mechanism involved.

In conclusion, the present work demonstrates that TBBPA exposure results in altered protamine ratios and increased sperm DNA fragmentation. The possibility is now open to further investigation of the molecular mechanisms within the sperm cell through which TBBPA exposure results in the detected effects. Different studies have demonstrated the presence of changes in the sperm proteome in infertile patients linked to protamine imbalances and DNA fragmentation (de Mateo *et al.*, 2007; Martinez-Heredia *et al.*, 2008; Oliva *et al.*, 2008; Amaral *et al.*, 2014). Therefore, important future extensions of our present work will be to explore in detail the overall sperm proteome and epigenome in TBBPA-exposed animals and their offspring, with the potential to provide additional important clues onto the mechanisms involved in the detected alterations. It will also be interesting in the future to conduct studies that limit the exposure of TBBPA to differing stages of development to gain further insight into the mechanisms of the detected associations. Another aspect that could be further investigated is to what extent the individual cell-to-cell differences in the protamine content, RNA and DNA fragmentation could relate to the detected associations in mixed sperm populations. Finally yet importantly, our results could also emphasize that more attention should be devoted to studying the potential health issues of TBBPA exposure in the human population and particularly those related to reproductive fitness.

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DISCLOSURES

The authors have nothing to disclose.

AUTHORS' CONTRIBUTION

EZ, JP and RO designed the study. EZ, JC, FE, AK and LD performed the bench work. EZ, JC, JP and RO interpreted the data. EZ, and RO wrote the manuscript. All authors critically reviewed and approved the submitted version of the manuscript.

REFERENCES

Amaral A, Castillo J, Ramalho-Santos J & Oliva R. (2014) The combined human sperm proteome: cellular pathways and implications for basic and clinical science. *Hum Reprod Update* 20, 40–62.

Aoki VW, Moskovtsev SI, Willis J, Liu L, Mullen JB & Carrell DT. (2005) DNA integrity is compromised in protamine-deficient human sperm. *J Androl* 26, 741–748.

Arpanahi A, Brinkworth M, Iles D, Krawetz SA, Paradowska A, Platts AE, Saida M, Steger K, Tedder P & Miller D. (2009) Endonuclease-sensitive regions of human spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences. *Genome Res* 19, 1338–1349.

Ausio J. (1992) Presence of a highly specific histone H1-like protein in the chromatin of the sperm of the bivalve mollusks. *Mol Cell Biochem* 115, 163–172.

Balhorn R. (2007) The protamine family of sperm nuclear proteins. *Genome Biol* 8, 227.
Cariou R, Antignac JP, Zalko D, Berrebi A, Cravedi JP, Maume D, Marchand P, Monteau F, Riu A, Andre F & Le Bizec B. (2008) Exposure assessment of French women and their newborns to tetrabromobisphenol-A: occurrence measurements in maternal adipose tissue, serum, breast milk and cord serum. *Chemosphere* 73, 1036–1041.
Carrell DT, Emery BR & Hammoud S. (2008) The aetiology of sperm protamine abnormalities and their potential impact on the sperm epigenome. *Int J Androl* 31, 537–545.
Castillo J, Simon L, de Mateo S, Lewis S & Oliva R. (2011) Protamine/DNA ratios and DNA damage in native and density gradient centrifuged sperm from infertile patients. *J Androl* 32, 324–332.
Castillo J, Amaral A & Oliva R. (2014) Sperm nuclear proteome and its epigenetic potential. *Andrology* 2, 326–338.
Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB & Eddy EM. (2001) Haploinsufficiency of protamine-1 or -2 causes infertility in mice. *Nat Genet* 28, 82–86.
Cho C, Jung-Ha H, Willis WD, Goulding EH, Stein P, Xu Z, Schultz RM, Hecht NB & Eddy EM. (2003) Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. *Biol Reprod* 69, 211–217.
Corzett M, Mazrimas J & Balhorn R. (2002) Protamine 1: protamine 2 stoichiometry in the sperm of eutherian mammals. *Mol Reprod Dev* 61, 519–527.
Domínguez-Fandos D, Camejo MI, Ballescà JL & Oliva R. (2007) Human sperm DNA fragmentation: correlation of TUNEL results as assessed by flow cytometry and optical microscopy. *Cytometry A* 71, 1011–1018.
Elzeinova F, Novakova V, Buckiova D, Kubatova A & Peknicova J. (2008) Effect of low dose of vinclozolin on reproductive tract development and sperm parameters in CD1 outbred mice. *Reprod Toxicol* 26, 231–238.
European Food Safety Authority (EFSA) (2011) Scientific opinion on tetrabromobisphenol A (TBBPA) and its derivatives in food. *EFSA J* 9, 2477.
Gardiner-Garden M, Ballesteros M, Gordon M & Tam PP. (1998) Histone- and protamine-DNA association: conservation of different patterns within the beta-globin domain in human sperm. *Mol Cell Biol* 18, 3350–3356.
Gatewood JM, Cook GR, Balhorn R, Bradbury EM & Schmid CW. (1987) Sequence-specific packaging of DNA in human sperm chromatin. *Science* 236, 962–964.
Guerrero-Bosagna C, Covert T, Haque M, Settles M, Nilsson E, Anway M & Skinner M. (2012) Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. *Reprod Toxicol* 34, 694–707.
Hammoud S, Liu L & Carrell DT. (2009) Protamine ratio and the level of histone retention in sperm selected from a density gradient preparation. *Andrologia* 41, 88–94.
Hammoud SS, Nix DA, Hammoud AO, Gibson M, Cairns BR & Carrell DT. (2011) Genome-wide analysis identifies changes in histone retention and epigenetic modifications at developmental and imprinted gene loci in the sperm of infertile men. *Hum Reprod* 26, 2558–2569.
Ihara M, Meyer-Ficca ML, Leu NA, Rao S, Li F, Gregory BD, Zalenskaya IA, Schultz RM & Meyer RG (2014) Paternal poly (ADP-ribose) metabolism modulates retention of inheritable sperm histones and early embryonic gene expression. *PLoS Genet* 10, e1004317.
Jakobsson K, Thuresson K, Rylander L, Sjodin A, Hagmar L & Bergman A. (2002) Exposure to polybrominated diphenyl ethers and tetrabromobisphenol A among computer technicians. *Chemosphere* 46, 709–716.
Jodar M & Oliva R. (2014) Protamine alterations in human spermatozoa. *Adv Exp Med Biol* 791, 83–102.
Jodar M, Kalko S, Castillo J, Ballesca JL & Oliva R. (2012) Differential RNAs in the sperm cells of asthenozoospermic patients. *Hum Reprod* 27, 1431–1438.
Kawashiro Y, Fukata H, Omori-Inoue M, Kubonoya K, Jotaki T, Takigami H, Sakai S & Mori C. (2008) Perinatal exposure to brominated flame retardants and polychlorinated biphenyls in Japan. *Endoc J* 55, 1071–1084.
Kitamura S, Jinno N, Ohta S, Kuroki H & Fujimoto N. (2002) Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. *Biochem Biophys Res Commun* 293, 554–559.
Korner W, Hanf V, Schuller W, Bartsch H, Zwirner M & Hagenmaier H. (1998) Validation and application of a rapid in vitro assay for assessing the estrogenic potency of halogenated phenolic chemicals. *Chemosphere* 37, 2395–2407.
Linhartova P, Gazo I, Shaliutina-Kolesova A & Hulak M Kaspar V (2014) Effects of tetrabrombisphenol A on DNA integrity, oxidative stress,and sterlet (Acipenserruthenus) spermatozoa quality variables. *Environ Toxicol*. doi: 10.1002/tox.21953 [Epub ahead of print].
Livak KJ & Schmittgen TD. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408.
Manikkam M, Tracey R, Guerrero-Bosagna C & Skinner M. (2013) Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *PLoS One* 8, e55387.
Martinez-Heredia J, de Mateo S, Vidal-Taboada J, Ballesca J & Oliva R. (2008) Identification of proteomic differences in asthenozoospermic sperm samples. *Hum Reprod* 23, 783–791.
de Mateo S, Martinez-Heredia J, Estanyol J, Domiguez-Fandos D, Vidal-Taboada J, Ballesca J & Oliva R. (2007) Marked correlations in protein expression identified by proteomic analysis of human spermatozoa. *Proteomics* 7, 4264–4277.
de Mateo S, Gazquez C, Guimera M, Balasch J, Meistrich ML, Ballesca JL & Oliva R. (2009) Protamine 2 precursors (Pre-P2), protamine 1 to protamine 2 ratio (P1/P2), and assisted reproduction outcome. *Fertil Steril* 91, 715–722.
de Mateo S, Ramos L, de Boer P, Meistrich M & Oliva R. (2011) Protamine 2 precursors and processing. *Protein Pept Lett* 18, 778–785.
Meerts IA, van Zanden JJ, Luijks EA, van Leeuwen-Bol I, Marsh G, Jakobsson E, Bergman A & Brouwer A. (2000) Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. *Toxicol Sci* 56, 95–104.
Mengual L, Ballesca JL, Ascaso C & Oliva R. (2003) Marked differences in protamine content and P1/P2 ratios in sperm cells from percoll fractions between patients and controls. *J Androl* 24, 438–447.
Oliva R. (2006) Protamines and male infertility. *Hum Reprod Update* 12, 417–435.
Oliva R & Ballesca JL. (2012) Altered histone retention and epigenetic modifications in the sperm of infertile men. *Asian J Androl* 14, 239–240.
Oliva R & Dixon GH. (1991) Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Prog Nucleic Acid Res Mol Biol* 40, 25–94.
Oliva R, Martinez-Heredia J & Estanyol JM. (2008) Proteomics in the study of the sperm cell composition, differentiation and function. *Syst Biol Reprod Med* 54, 23–36.
Oliva R & Castillo J. (2011) Sperm nucleoproteins. In: *Sperm chromatin: biological and clinical applications in male infertility and assisted reproduction* (eds A Zini & A Agarwal), pp. 45–60. Springer, New York.
Rochester J. (2013) Bisphenol A and human health: a review of the literature. *Reprod Toxicol* 42, 132–155.
Samuelsen M, Olsen C, Holme JA, Meussen-Elholm E, Bergmann A & Hongslo JK. (2001) Estrogen-like properties of brominated analogs of bisphenol A in the MCF-7 human breast cancer cell line. *Cell Biol Toxicol* 17, 139–151.

Sellstrom U & Jansson B. (1995) Analysis of tetrabromobisphenol A in a product and environmental samples. *Chemosphere* 31, 3085–3092.
Simon L, Castillo J, Oliva R & Lewis SE. (2011) Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. *Reprod Biomed Online* 23, 724–734.
Steger K, Pauls K, Klonisch T, Franke FE & Bergmann M. (2000) Expression of protamine-1 and -2 mRNA during human spermiogenesis. *Mol Hum Reprod* 6, 219–225.
Steger K, Failing K, Klonisch T, Behre HM, Manning M, Weidner W, Hertle L, Bergmann M & Kliesch S. (2001) Round spermatids from infertile men exhibit decreased protamine-1 and -2 mRNA. *Hum Reprod* 16, 709–716.
Steger K, Fink L, Failing K, Bohle RM, Kliesch S, Weidner W & Bergmann M. (2003) Decreased protamine-1 transcript levels in testes from infertile men. *Mol Hum Reprod* 9, 331–336.
Takigami H, Suzuki G, Hirai Y & Sakai S. (2009) Brominated flame retardants and other polyhalogenated compounds in indoor air and dust from two houses in Japan. *Chemosphere* 76, 270–277.
Tomsu M, Sharma V & Miller D. (2002) Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters. *Hum Reprod* 17, 1856–1862.
Torregrosa N, Domínguez-Fandos D, Camejo MI, Shirley CR, Meistrich ML, Ballescà JL & Oliva R. (2006) Protamine 2 (P2) precursors, P1/P2 ratio, DNA integrity and other sperm parameters in infertile patients. *Hum Reprod* 21, 2084–2089.
Uhnakova B, Ludwig R, Peknicova J, Homolka L, Lisa L, Sulc M, Petrickova A, Elzeinova F, Pelantova H, Monti D, Kren D, Haltrich D & Martinkova L. (2011) Biodegradation of tetrabromobisphenol A by oxidases in basidiomycetous fungi and estrogenic activity of the biotransformation products. *Bioresour Technol* 102, 9409–9415.
Van der Ven LT, Van de Kuil T, Verhoef A, Verwer CM, Lilienthal H, Leonards PE, Schauer UM, Canton RF, Litens S, De Jong FH, Visser TJ, Dekant W, Stern N, Hakansson H, Slob W, Van den Berg M, Vos JG & Piersma AH. (2008) Endocrine effects of tetrabromobisphenol-A (TBBPA) in Wistar rats as tested in a one-generation reproduction study and a subacute toxicity study. *Toxicology* 245, 76–89.
de Wit CA. (2002) An overview of brominated flame retardants in the environment. *Chemosphere* 46, 583–624.
Wu SF, Zhang H & Cairns BR. (2011) Genes for embryo development are packaged in blocks of multivalent chromatin in zebrafish sperm. *Genome Res* 21, 578–589.
Wykes SM & Krawetz SA. (2003) The structural organization of sperm chromatin. *J Biol Chem* 278, 29471–29477.
de Yebra L & Oliva R. (1993) Rapid analysis of mammalian sperm nuclear proteins. *Anal Biochem* 209, 201–203.
Zalenskaya IA, Bradbury EM & Zalensky AO. (2000) Chromatin structure of telomere domain in human sperm. *Biochem Biophys Res Commun* 279, 213–218.
Zatecka E, Ded L, Elzeinova F, Kubatova A, Dorosh A, Margaryan H, Dostalova P & Peknicova J. (2013) Effect of tetrabrombisphenol A on induction of apoptosis in the testes and changes in expression of selected testicular genes in CD1 mice. *Reprod Toxicol* 35, 32–39.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. General reproductive parameters and body weight in the control and TBBPA groups

3.3.

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

Žatecká E., Děd L., Elzeinová F., Kubátová A., Dorosh A., Margaryan H., Dostálová P., Korenková V., Hošková K. and Pěknicová J.

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

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

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

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

2. Materials and methods

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

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

2.2. Anogenital distance, body and organ weight

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

2.3. Preparation of cells

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

2.4. Sperm viability

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

2.5. Sperm morphology

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

2.6. Indirect immunofluorescence

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

2.7. Apoptotic stage of spermatozoa

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

2.8. Histological analysis

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

2.9. TUNEL analysis

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

2.10. RNA isolation

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

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

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

2.12. Quantitative polymerase chain reaction (qPCR)

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

Table 1
Primer sequences.

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

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

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

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

2.13. Selected testicular genes

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

2.14. Statistical analysis

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

3. Results

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

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

3.2. Sperm parameters

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

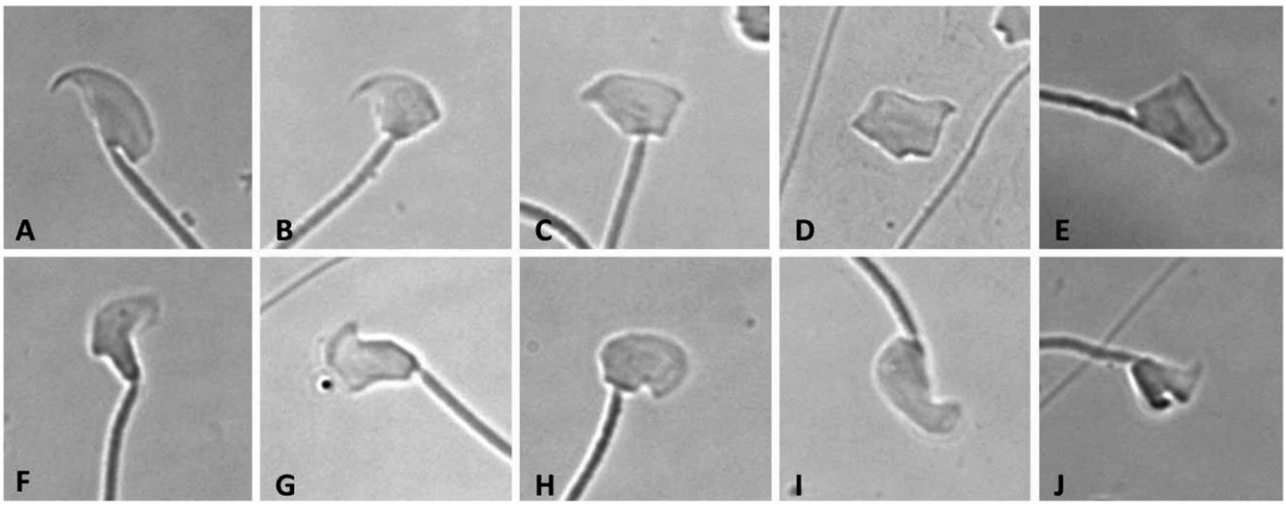

Fig. 1. Exemplary pictures from light microscopy showing sperm with normal (A) and aberrant morphology (B–J).

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

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

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

3.3. Histology of testes

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

3.4. TUNEL analysis

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

3.5. Real-time PCR of testicular genes

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

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

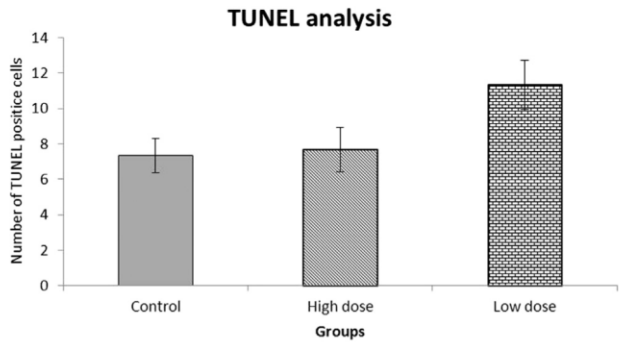

Fig. 2. Number of TUNEL-positive cells in testicular tissue sections. The high-dose group was exposed to 150 μg/l of ZE; the low-dose group was exposed to 0.15 μg/l of ZE. Mean ± SD; $n = 18$.

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

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

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

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

4. Discussion

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

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

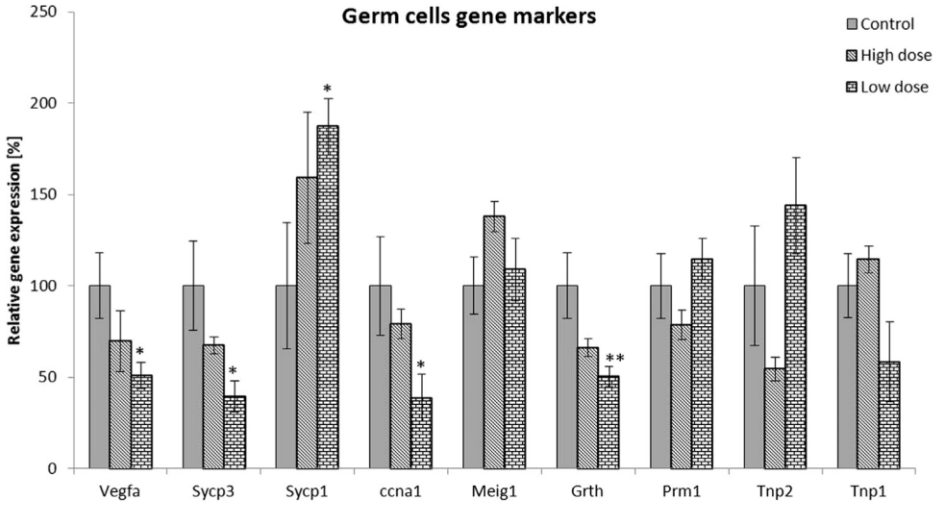

Fig. 3. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes expressed in testicular germ cells. In this study, genes for vascular endothelial growth factor (*Vegfa*), synaptonemal complex protein 3 (*Sycp3*), synaptonemal complex protein 1 (*Sycp1*), cyclin A1 (*Ccna1*),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

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

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

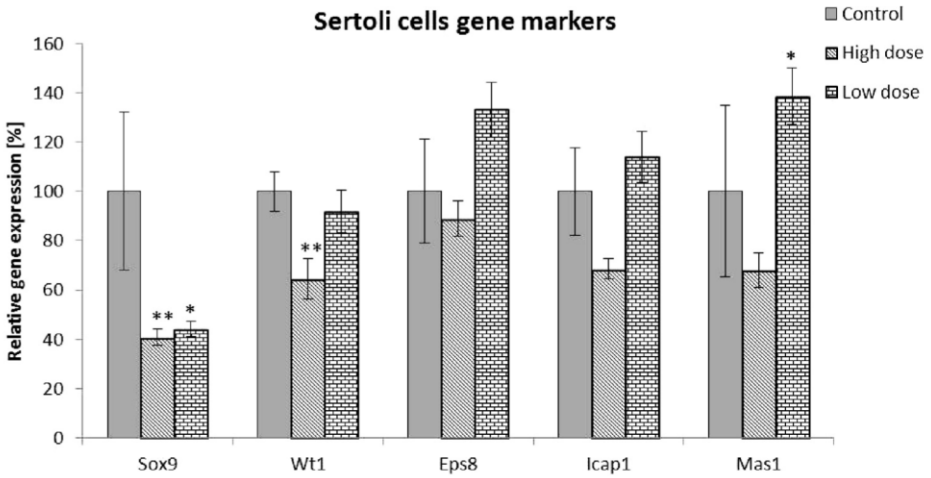

Fig. 4. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes expressed in Sertoli cells. In this study, genes for SRY-box containing gene 9 (*Sox9*), Wilms tumour 1 (*Wt1*), epidermal growth factor receptor pathway substrate 8 (*Eps8*), integrin beta 1 binding protein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

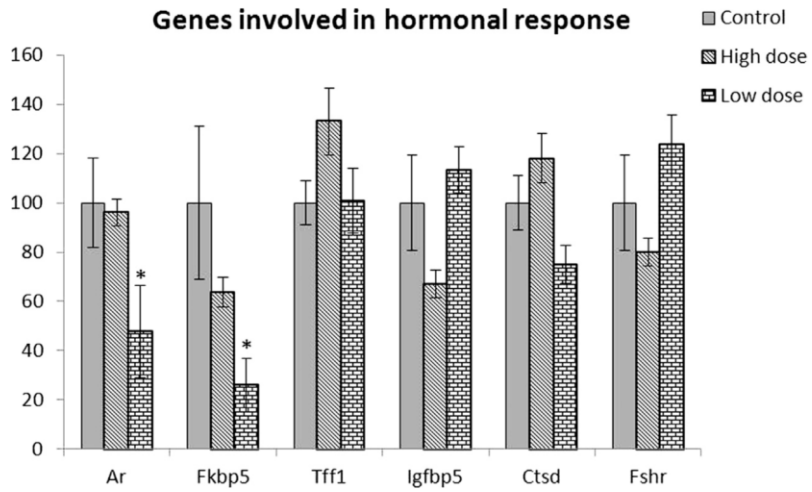

Fig. 5. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes playing a role in the response to various hormonal stimuli. In this study, genes for androgen receptor (*Ar*), FK506 binding protein 5 (*Fkbp5*), trefoil factor 1 (*Tff1*), insulin-like growth factor binding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

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

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

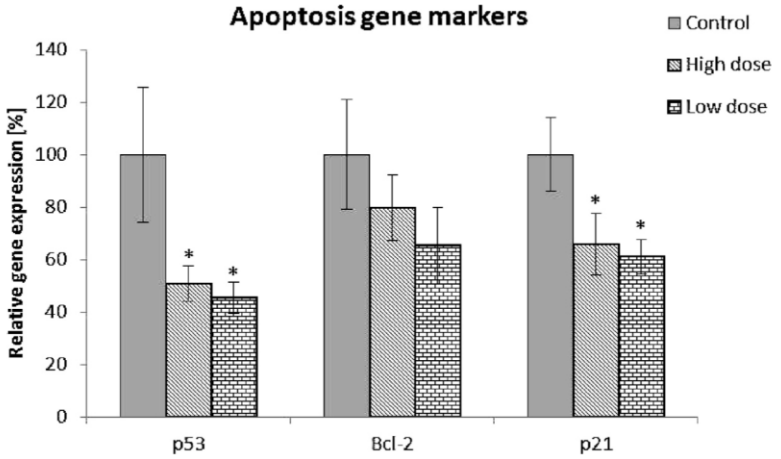

Fig. 6. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes playing a role in apoptosis. In this study, genes for tumour protein p53 (*p53*), B-cell leukaemia/lymphoma 2 (*Bcl2*) and cyclin-dependent kinase inhibitor 1A (*p21*) were tested. The graph illustrates the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

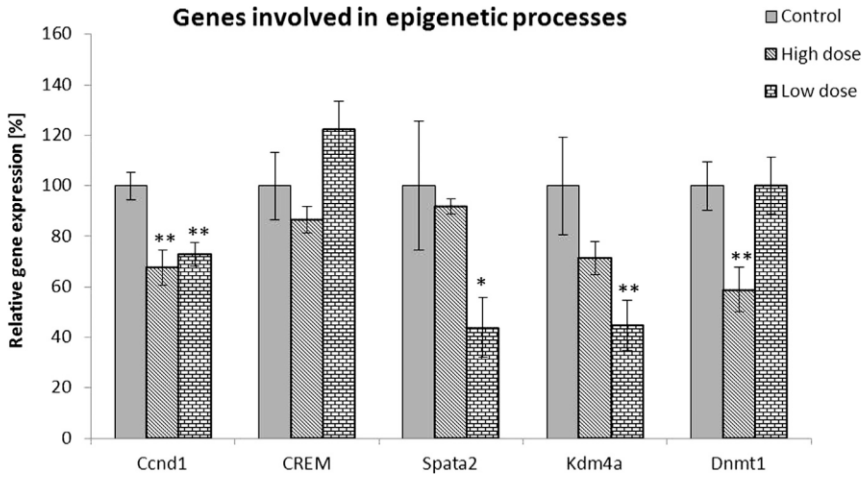

Fig. 7. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes that are related to epigenetic processes. In this study, genes for cyclin D1 (*Ccnd1*), cAMP responsive element modulator (*Crem*), lysine (K)-specific demethylase 4A (*Kdm4a*), spermatogenesis associated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

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

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

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

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

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

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

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

5. Conclusion

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

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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

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References

[1] Zinedine A, Soriano JM, Molto JC, Manes J. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association 2007;45(1):1–18.

[2] E.F.S.A. Panel on Contaminants in the Food Chain (CONTAM): scientific opinion on the risks for public health related to the presence of zearalenone in food. EFSA Journal 2011;9(6):2197.
[3] Waskiewicz A, Gromadzka K, Bocianowski J, Pluta P, Golinski P. Zearalenone contamination of the aquatic environment as a result of its presence in crops. Arhiv za Higijenu Rada i Toksikologiju 2012;63:429–35.
[4] Prelusky DB, Scott PM, Trenholm HL, Lawrence GA. Minimal transmission of zearalenone to milk of dairy cows. Journal of Environmental Science and Health Part B: Pesticides, Food Contaminants, and Agricultural Wastes 1990;25(1):87–103.
[5] Olsen M, Pettersson H, Sandholm K, Visconti A, Kiessling KH. Metabolism of zearalenone by sow intestinal mucosa in vitro. Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association 1987;25(9):681–3.
[6] Biehl ML, Prelusky DB, Koritz GD, Hartin KE, Buck WB, Trenholm HL. Biliary excretion and enterohepatic cycling of zearalenone in immature pigs. Toxicology and Applied Pharmacology 1993;121(1):152–9.
[7] Kuiper-Goodman T, Scott PM, Watanabe H. Risk assessment of the mycotoxin zearalenone. Regulatory Toxicology and Pharmacology 1987;7(3):253–306.
[8] European Commission Opinion of the Scientific Committee on food on Fusarium Toxins: Part 2: Zearalenone (ZEA) [expressed June 22] http://ec.europa.eu/food/fs/sc/scf/out65_en.pdf, expressed June 22, 2000.
[9] Yang J, Zhang Y, Wang Y, Cui S. Toxic effects of zearalenone and alpha-zearalenol on the regulation of steroidogenesis and testosterone production in mouse Leydig cells. Toxicology In Vitro 2007;21:558–65.
[10] Yang JY, Wang GX, Liu JL, Fan JJ, Cui S. Toxic effects of zearalenone and its derivatives alpha-zearalenol on male reproductive system in mice. Reproductive Toxicology 2007;24:381–7.
[11] Kim IH, Son HY, Cho SW, Ha CS, Kang BH. Zearalenone induces male germ cell apoptosis in rats. Toxicology Letters 2003;138:185–92.
[12] Yuan H, Deng Y, Yuan L, Wu J, Yuan Z. Gynostemma pentaphyllum protects mouse male germ cells against apoptosis caused by zearalenone via Bax and Bcl-2 regulation. Toxicological Mechanism and Methods 2010;20:153–8.
[13] Filipiak E, Walczak-Jedrzejowska R, Oszukowska E, Guminska A, Marchlewska K. Xenoestrogens diethylstilbestrol and zearalenone negatively influence pubertal rat's testis. Folia Histochemica et Cytobiologica 2009;47:S113–20.
[14] Peknicova J, Kyselova V, Buckiova D, Boubelik M. Effect of an endocrine disruptor on mammalian fertility. Application of monoclonal antibodies against sperm proteins as markers for testing sperm damage. American Journal of Reproductive Immunology 2002;47(5):311–8.
[15] Peknicova J, Chladek D, Hozak P. Monoclonal antibodies against sperm intra-acrosomal antigens as markers for male infertility diagnostics and estimation of spermatogenesis. American Journal of Reproductive Immunology 2005;53(1):42–9.
[16] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25(4):402–8.
[17] Lopez-Casas PP, Mizrak SC, Lopez-Fernandez LA, Paz M, de Rooij DG, del Mazo J. The effects of different endocrine disruptors defining compound-specific alterations of gene expression profiles in the developing testis. Reproductive Toxicology 2012;33:106–15.
[18] Lee YM, Rhee JS, Hwang DS, Kim IC, Raisuddin S, Lee JS. p53 gene expression is modulated by endocrine disrupting chemicals in the hermaphroditic fish, *Kryptolebias marmoratus*. Comparative Biochemistry and Physiology Toxicology & Pharmacology 2008;147(2):150–7.
[19] Caires KC, de Avila JM, Cupp AS, McLean DJ. VEGFA family isoforms regulate spermatogonial stem cell homeostasis in vivo. Endocrinology 2012;153(2):887–900.
[20] Caires KC, de Avila J, McLean DJ. Vascular endothelial growth factor regulates germ cell survival during establishment of spermatogenesis in the bovine testis. Reproduction 2009;138(4):667–77.
[21] Zheng YH, Rengaraj D, Choi JW, Park KJ, Lee SI, Han JY. Expression pattern of meiosis associated SYCP family members during germline development in chickens. Reproduction 2009;138(3):483–92.
[22] Bisig CG, Guiraldelli MF, Kouznetsova A, Scherthan H, Hoog C, Dawson DS, et al. Synaptonemal complex components persist at centromeres and are required for homologous centromere pairing in mouse spermatocytes. PLoS Genetics 2012;8(6):e1002701.
[23] Liu D, Matzuk MM, Sung WK, Guo Q, Wang P, Wolgemuth DJ. Cyclin A1 is required for meiosis in the male mouse. Nature Genetics 1998;20(4):377–80.
[24] van der Meer T, Chan WY, Palazon LS, Nieduszynski C, Murphy M, Sobczak-Thepot J, et al. Cyclin A1 protein shows haplo-insufficiency for normal fertility in male mice. Reproduction 2004;127(4):503–11.
[25] Dufau ML, Tsai-Morris CH. Gonadotropin-regulated testicular helicase (GRTH/DDX25): an essential regulator of spermatogenesis. Trends in Endocrinology and Metabolism 2007;18(8):314–20.
[26] Frojdman K, Harley VR, Pelliniemi LJ. Sox9 protein in rat sertoli cells is age and stage dependent. Histochemistry and Cell Biology 2000;113(1):31–6.
[27] Bettegowda A, Wilkinson MF. Transcription and post-transcriptional regulation of spermatogenesis. Philosophical Transactions of the Royal Society of London Series B, Biological Sciences 2010;365(1546):1637–51.
[28] Xu P, Santos RA, Bader M, Alenina N. Alterations in gene expression in the testis of angiotensin-(1-7)-receptor Mas-deficient mice. Regulatory Peptides 2007;138(2/3):51–5.
[29] Trasler JM. Epigenetics in spermatogenesis. Molecular and Cellular Endocrinology 2009;306(1/2):33–6.
[30] Okada Y, Scott G, Ray MK, Mishina Y, Zhang Y. Histone demethylase JHDM2A is critical for Tnp1 and Prm1 transcription and spermatogenesis. Nature 2007;450(7166):119–23.
[31] Yamane K, Toumazou C, Tsukada Y, Erdjument-Bromage H, Tempst P, Wong J, et al. JH.DM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell 2006;125(3):483–95.
[32] Onisto M, Slongo LM, Graziotto R, Zotti L, Negro A, Merico M, et al. Evidence for FSH-dependent upregulation of SPATA2 (spermatogenesis-associated protein 2). Biochemical and Biophysical Research Communications 2001;283(1):86–92.
[33] Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Proliferation 2003;36(3):131–49.
[34] Stouder C, Paoloni-Giacobino A. Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. Reproduction 2010;139(2):373–9.
[35] Marques CJ, Francisco T, Sousa S, Carvalho F, Barros A, Sousa M. Methylation defects of imprinted genes in human testicular spermatozoa. Fertility and Sterility 2010;94(2):585–94.
[36] Minervini F, Giannoccaro A, Cavallini A, Visconti A. Investigations on cellular proliferation induced by zearalenone and its derivatives in relation to the estrogenic parameters. Toxicology Letters 2005;159(3):272–83.
[37] Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998;139(10):4252–63.
[38] Jefferson WN, Padilla-Banksa E, Clark G, Newbolda RR. Assessing estrogenic activity of phytochemicals using transcriptional activation and immature mouse uterotrophic responses. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences 2002;777(1/2):179–89.
[39] Takemura H, Shim JY, Sayama K, Tsubura A, Zhu BT, Shimoi K. Characterization of the estrogenic activities of zearalenone and zeranol in vivo and in vitro. Journal of Steroid Biochemistry and Molecular Biology 2007;103(2):170–7.
[40] Heneweer M, Houtman R, Poortman J, Groot M, Maliepaar C, Peijnenburg A. Estrogenic effects in the immature rat uterus after dietary exposure to ethinylestradiol and zearalenone using a systems biology approach. Toxicological Sciences: An Official Journal of the Society of Toxicology 2007;99(1):303–14.
[41] Minervini F, Dell'Aquila ME. Zearalenone and reproductive function in farm animals. International Journal of Molecular Sciences 2008;9(12):2570–84.
[42] Collins TF, Sprando RL, Black TN, Olejnik N, Eppley RM, Alam HZ, et al. Effects of zearalenone on in utero development in rats. Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association 2006;44.(9).
[43] Appelgren LE, Arora RG, Larsson P. Autoradiographic studies of [3H] zearalenone in mice. Toxicology 1982;25(2-3):243–53.
[44] Richter CA, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, et al. In vivo effects of bisphenol A in laboratory rodent studies. Reproductive Toxicology 2007;24(2):199–224.
[45] Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, et al. In vitro molecular mechanisms of bisphenol A action. Reproductive Toxicology 2007;24(2):178–98.
[46] Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environmental Health Perspective 2003;111:994–1006.
[47] Fox TO. Androgen- and estrogen-binding macromolecules in developing mouse brain: biochemical and genetic evidence. Proceedings of the National Academy of Sciences of the USA 1975;72:4303–7.

3.4

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

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REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY

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

Andriy Dorosh[1], Olina Tepla[2], Eva Zatecka[1], Lukas Ded[1], Karel Koci[2] and Jana Peknicova[1*]

Abstract

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

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

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

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

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

Background

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

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

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

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

Methods

Patients

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

TESE procedure, sperm extraction and ICSI

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

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

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

RNA purification

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

RT-qPCR

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

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

Statistical analysis

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

Results

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

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

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

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

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

Discussion

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

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

Table 1 Primer sequences

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

Table 2 Fertilization outcomes in individual patients

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

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

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

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

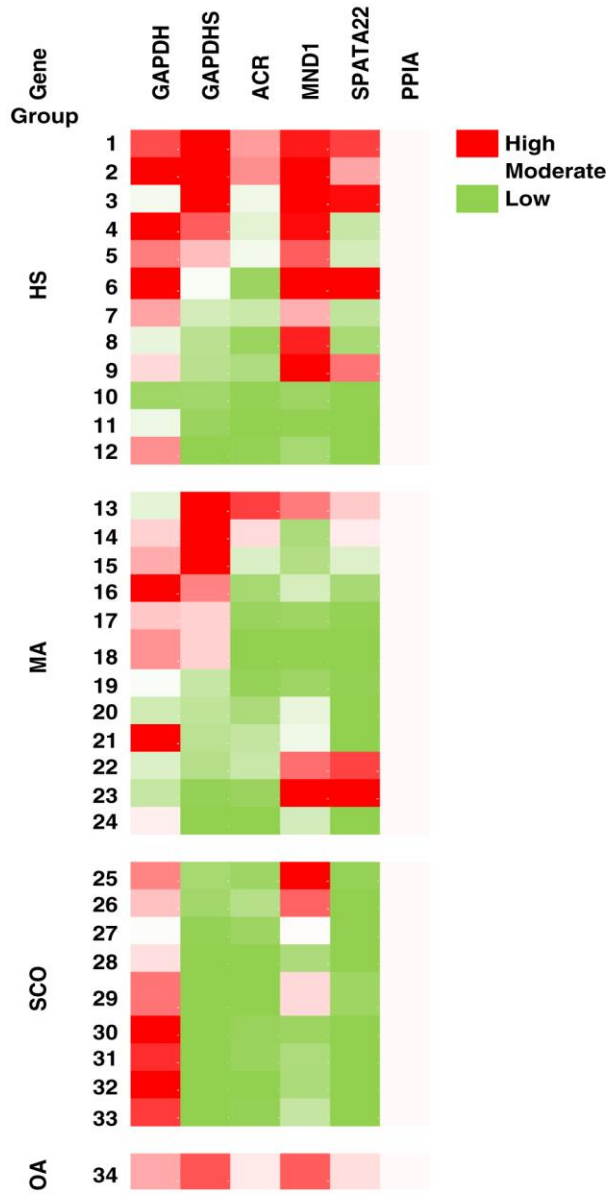

Figure 1 Heat map representing the differential gene expression of the tested genes among individual samples and groups. Red color indicates the highest gene expression (90th percentile), white color indicates moderated gene expression and green color indicates lowest gene expression (10% percentile).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

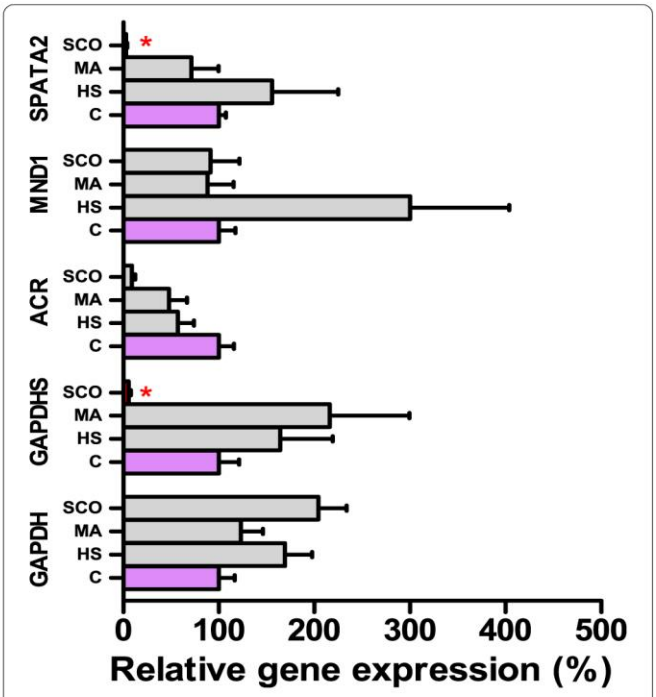

Figure 2 The relative difference in the expression of tested genes among control and experimental groups of hypospermatogenesis (HS), maturation arrest (MA) and Sertoli cell- only syndrome (SCO). Columns indicate the arithmetic mean of the relative expression of a particular gene, whiskers indicate SEM.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

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

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

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

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

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

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

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

Conclusions

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

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

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

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References

1. Shima JE, McLean DJ, McCarrey JR, Griswold MD: **The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis.** *Biol Reprod* 2004, **71**:319–330.
2. Gatta V, Raicu F, Ferlin A, Antonucci I, Scioletti AP, Garolla A, Palka G, Foresta C, Stuppia L: **Testis transcriptome analysis in male infertility: new insight on the pathogenesis of oligo-azoospermia in cases with and without AZFc microdeletion.** *BMC Genomics* 2010, **11**:401.

3. Calvel P, Rolland AD, Jegou B, Pineau C: **Testicular postgenomics: targeting the regulation of spermatogenesis.** *Philos Trans R Soc Lond B Biol Sci* 2010, **365:**1481–1500.
4. Okada H, Tajima A, Shichiri K, Tanaka A, Tanaka K, Inoue I: **Genome-wide expression of azoospermia testes demonstrates a specific profile and implicates ART3 in genetic susceptibility.** *PLoS Genet* 2008, **4:**e26.
5. Ellis PJ, Furlong RA, Conner SJ, Kirkman-Brown J, Afnan M, Barratt C, Griffin DK, Affara NA: **Coordinated transcriptional regulation patterns associated with infertility phenotypes in men.** *J Med Genet* 2007, **44:**498–508.
6. Rockett JC, Patrizio P, Schmid JE, Hecht NB, Dix DJ: **Gene expression patterns associated with infertility in humans and rodent models.** *Mutat Res* 2004, **549:**225–240.
7. Tepla O, Peknicova J, Koci K, Mika J, Mrazek M, Elzeinova F: **Evaluation of reproductive potential after intracytoplasmic sperm injection of varied human semen tested by antiacrosomal antibodies.** *Fertil Steril* 2006, **86:**113–120.
8. Holstein AF, Schulze W, Davidoff M: **Understanding spermatogenesis is a prerequisite for treatment.** *Reprod Biol Endocrinol* 2003, **1:**107.
9. Silber SJ, Van Steirteghem AC, Liu J, Nagy Z, Tournaye H, Devroey P: **High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy.** *Hum Reprod* 1995, **10:**148–152.
10. 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* 2012, **4:**4.
11. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL: **Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction.** *BMC Bioinformatics* 2012, **13:**134.
12. Liu YJ, Zheng D, Balasubramanian S, Carriero N, Khurana E, Robilotto R, Gerstein MB: **Comprehensive analysis of the pseudogenes of glycolytic enzymes in vertebrates: the anomalously high number of GAPDH pseudogenes highlights a recent burst of retrotrans-positional activity.** *BMC Genomics* 2009, **10:**480.
13. Barber RD, Harmer DW, Coleman RA, Clark BJ: **GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues.** *Physiol Genomics* 2005, **21:**389–395.
14. Vernaeve V, Tournaye H, Schiettecatte J, Verheyen G, Steirteghem AV, Devroey P: **Serum inhibin B cannot predict testicular sperm retrieval in patients with non-obstructive azoospermia.** *Hum Reprod* 2002, **17:**971–976.
15. Tsujimura A, Matsumiya K, Miyagawa Y, Takao T, Fujita K, Koga M, Takeyama M, Fujioka H, Okuyama A: **Prediction of successful outcome of microdissection testicular sperm extraction in men with idiopathic nonobstructive azoospermia.** *J Urol* 2004, **172:**1944–1947.
16. Samli MM, Dogan I: **An artificial neural network for predicting the presence of spermatozoa in the testes of men with nonobstructive azoospermia.** *J Urol* 2004, **171:**2354–2357.
17. Ezeh UIO, Taub NA, Moore HDM, Cooke ID: **Establishment of predictive variables associated with testicular sperm retrieval in men with non-obstructive azoospermia.** *Hum Reprod* 1999, **14:**1005–1012.
18. Divina P, Vlcek C, Strnad P, Paces V, Forejt J: **Global transcriptome analysis of the C57BL/6J mouse testis by SAGE: evidence for nonrandom gene order.** *BMC Genomics* 2005, **6:**29.
19. Fox MS, Ares VX, Turek PJ, Haqq C, Reijo Pera RA: **Feasibility of global gene expression analysis in testicular biopsies from infertile men.** *Mol Reprod Dev* 2003, **66:**403–421.
20. Chalmel F, Rolland AD, Niederhauser-Wiederkehr C, Chung SS, Demougin P, Gattiker A, Moore J, Patard JJ, Wolgemuth DJ, Jegou B, Primig M: **The conserved transcriptome in human and rodent male gametogenesis.** *Proc Natl Acad Sci USA* 2007, **104:**8346–8351.
21. Schultz N, Hamra FK, Garbers DL: **A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets.** *Proc Natl Acad Sci USA* 2003, **100:**12201–12206.
22. Lian J, Zhang X, Tian H, Liang N, Wang Y, Liang C, Li X, Sun F: **Altered microRNA expression in patients with non-obstructive azoospermia.** *Reprod Biol Endocrinol* 2009, **7:**1477–7827.
23. Lee JH, Lee DR, Yoon SJ, Chai YG, Roh SI, Yoon HS: **Expression of DAZ (deleted in azoospermia), DAZL1 (DAZ-like) and protamine-2 in testis and its application for diagnosis of spermatogenesis in non-obstructive azoospermia.** *Mol Hum Reprod* 1998, **4:**827–834.
24. Lin YM, Kuo PL, Lin YH, Teng YN, Nan Lin JS: **Messenger RNA transcripts of the meiotic regulator BOULE in the testis of azoospermic men and their application in predicting the success of sperm retrieval.** *Hum Reprod* 2005, **20:**782–788.
25. Ando M, Yamaguchi K, Chiba K, Miyake H, Fujisawa M: **Expression of VASA mRNA in testis as a significant predictor of sperm recovery by microdissection testicular sperm extraction in patient with nonobstructive azoospermia.** *J Androl* 2012, **33:**711–716.
26. Tsubouchi H, Roeder GS: **The Mnd1 protein forms a complex with hop2 to promote homologous chromosome pairing and meiotic double-strand break repair.** *Mol Cell Biol* 2002, **22:**3078–3088.
27. La Salle S, Palmer K, O'Brien M, Schimenti JC, Eppig J, Handel MA: **Spata22, a novel vertebrate-specific gene, is required for meiotic progress in mouse germ cells.** *Biol Reprod* 2012, **86**(2):1–12.
28. Adham IM, Klemm U, Maier WM, Hoyer-Fender S, Tsaousidou S, Engel W: **Molecular cloning of preproacrosin and analysis of its expression pattern in spermatogenesis.** *Eur J Biochem* 1989, **182:**563–568.
29. Baba T, Azuma S, Kashiwabara S, Toyoda Y: **Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization.** *J Biol Chem* 1994, **269:**31845–31849.
30. Welch JE, Brown PL, O'Brien DA, Magyar PL, Bunch DO, Mori C, Eddy EM: **Human glyceraldehyde 3-phosphate dehydrogenase-2 gene is expressed specifically in spermatogenic cells.** *J Androl* 2000, **21:**328–338.
31. Welch JE, Barbee RR, Magyar PL, Bunch DO, O'Brien DA: **Expression of the spermatogenic cell-specific glyceraldehyde 3-phosphate dehydrogenase (GAPDS) in rat testis.** *Mol Reprod Dev* 2006, **73:**1052–1060.
32. Yang J, Chennathukuzhi V, Miki K, O'Brien DA, Hecht NB: **Mouse testis brain RNA-binding protein/translin selectively binds to the messenger RNA of the fibrous sheath protein glyceraldehyde 3-phosphate dehydrogenase-S and suppresses its translation in vitro.** *Biol Reprod* 2003, **68:**853–859.
33. Li H, Wu C, Gu X, Xiong C: **A novel application of cell-free seminal mRNA: non-invasive identification of the presence of germ cells or complete obstruction in men with azoospermia.** *Hum Reprod* 2012, **27:**991–997.
34. Aslani F, Modarresi MH, Soltanghoraee H, Akhondi MM, Shabani A, Lakpour N, Sadeghi MR: **Seminal molecular markers as a non-invasive diagnostic tool for the evaluation of spermatogenesis in non-obstructive azoospermia.** *Syst Biol Reprod Med* 2011, **57:**190–196.

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4. Summary of published results

In the following chapter I would like to highlight and discuss the main scientific outcome of the presented research papers.

4.1. The effect of TBBPA on male gonadal function with focus on testicular morphology, sperm quality and expression of selected genes. (Research papers 3.1. and 3.2.)

In these studies, we analyzed the effect of brominated flame retardant tetrabromobispehol A (TBBPA) on reproductive parameters of male mice *in vivo*. This environmental pollutant has been shown to be ubiquitous in the environment. The main source of contaminations are factories where TBBPA is produced, and it has also been shown that it is released from various electronic products and thus contaminating our homes or offices (Alaee et al., 2003; Takigami et al., 2009).

We conducted two-generational *in vivo* study where animals were continuously exposed to TBBPA. TBBPA was dissolved in drinking water in concentration 200 µg/l, which roughly corresponds to 35 µg/kg bw. In the 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 the adulthood. In group **C**, the pups were not exposed to TBBPA at all. Animals of F1 generation were bred at the age of 70 days to form F2 generation. The breeding was performed as follows – females and males from group **C** formed group **CC** (control group of F2 generation); both parents from group **T** formed group **TT** (interbreeding), female from group **C** and male from group **T** formed group **CT** (outcrossing), and female from group **T** and male from group **C** formed group **TC** (reverse outcrossing). Pups of F2 generation were exposed to TBBPA only in groups **TC** and **TT** (groups CC and CT were not exposed).

At the age of 70 days, animals of both generations were sacrificed and subjected to analysis. First, we evaluated the general reproductive parameters. We did not observe any effect of TBBPA on the number or sex ratio of the progeny, body weight and anogenital distance in both generations. Regarding the weight of reproductive organs, the most affected group was the group TT of F2 generation, where we noticed significantly reduced testicular weight and increased weight of the prostate and seminal vesicles. Evaluation of

sperm parameters (sperm morphology, viability and acrosome status) did not reveal any negative changes after exposure to TBBPA in both generations.

We also performed histological analysis using testis paraffin sections stained by hematoxylin (nucleus) and eosin (cytoplasm). In experimental groups we did not observe any visible pathological changes in morphology of seminiferous tubules. Likewise, the process of spermatogenesis was not interrupted. We also analyzed the epithelial thickness and tubule diameter. TBBPA did not have any effect on the tubule diameter; however, epithelial thickness was significantly lower in groups T (F1 generation) and TC; TT (F2 generation). To identify the number of apoptotic cells in the testes we performed the TUNEL analysis of testis paraffin sections. We observed increased numbers of apoptotic cells in the testes in group T of the F1 generation (almost double compared to the control) and in groups TC and TT of the F2 generation (about 25 % more apoptotic cells compared to the control).

To evaluate the effect of TBBPA on events taking place in testicular tissue more closely, we analyzed the expression profile of several testicular genes. The gene for proacrosin (Acr) was tested as an acrosome-specific gene. The gene for androgen receptor (Ar) was tested as an androgen-responsive gene. Several genes for heat shock proteins (Hsps) – Hsp70-2, Hsc70t, Hsp60, and APG-1 – were analyzed to determine the overall stress effect of TBBPA on testicular cells. Genes for Bax and Bcl-2 proteins were selected for their relation to apoptosis, and finally the Sox9 gene was chosen as a marker for Sertoli cells. The most affected groups were those that were exposed to TBBPA – groups T (F1 generation) and TC; TT (F2 generation), where we detected decreased expression of genes for Hsp70-2, Hsp60, Bcl-2 (anti-apoptotic protein) and Sox9. In these groups, we noticed increased expression of genes for Hsp70-t and Bax (pro-apoptotic protein). Moreover, we detected significantly decreased expression of the gene for Ar in groups TC and TT. Interestingly, we also observed certain changes of gene expression in group CT (F2 generation), which had not been exposed to TBBPA (only the father was). Here, we observed increased expression of genes for Hsp70-2 and Hsp60.

In summary, our results provide evidence that TBBPA is able to induce apoptosis of testicular cells, which is probably the cause of decreased thickness of seminiferous

epithelium. The analyzed sperm parameters did not show any alterations after TBBPA exposure; however, real-time PCR analysis revealed changes in the expression of selected testicular genes. These genes were selected because of their essential role during spermatogenesis, and thus any alteration of their expression might lead to impairment of this process. Our results also suggest that permanent exposure to TBBPA slightly enhances its effect in the next generation, depending on whether the parents were affected or not.

In our next study, we decided to get a closer look on the effect of TBBPA on spermatozoa as the carrier of parental genetic information. We analyzed the protamine content and DNA integrity in the mouse sperm after TBBPA exposure. In this study we used the same TBBPA concentration (200 µg/l) and treatment (continuous exposure during gestation, lactation, pre-pubertal, pubertal periods up to adulthood) as in previous study. Regarding the results from protamine analysis, we detected a decreased P1/P2 ratio (0.362 ± 0.024 in TBBPA group vs. 0.494 ± 0.052 in controls) and increased total protamine/DNA ratio (0.517 ± 0.073 in TBBPA vs. 0.324 ± 0.081 in controls). To analyze the sperm DNA integrity, we performed the TUNEL assay. In animals exposed to TBBPA, we observed higher number of TUNEL positive spermatozoa compared to the control (39.5 ± 4.5 % vs 21.2 ± 3.1 %), indicating higher sperm DNA fragmentation in the exposed animals. One of the main function of protamines is to protect the paternal genetic information (Carrell et al., 2007), and thus it is not surprising that in animals with altered sperm protamination we also observed higher sperm DNA fragmentation. Various studies have demonstrated the link of protamine imbalances and DNA fragmentation with male infertility (Carrell and Liu, 2001; Mengual et al., 2003; Aoki et al., 2005; Oliva, 2006). However, protamines might also be involved in other aspects than protection of paternal DNA. It has been shown that protamines play an important role in the sperm epigenome (Hammoud et al., 2009; Hammoud et al., 2011), and thus differential protamination of the sperm DNA may represent a possible mechanism of trans-generational transmission of the pathological phenotypes induced by environmental pollutants.

4.2. The effect of treatment with a low dose of mycotoxin zearalenone on the male gonadal function, sperm quality and expression of important testicular genes. (Research paper 3.3.)

Zearalenone (ZEA) is a nonsteroidal estrogenic mycotoxin produced by a variety of Fusarium fungi, which are common contaminants of cereal crops worldwide (Kuiper-Goodman et al., 1987). In this study, we investigated the effect of two different concentrations (150 μg/l and 0.15 μg/l) of ZEA on the reproductive parameters and expression of testicular genes in male mice *in vivo*. In this experiment, there were two experimental groups (one exposed to ZEA in concentration 0.15 μg/l and one at 150 μg/l) and one control group (not exposed). ZEA was administered in drinking water starting from the first day of mothers' pregnancy; the born pups were then exposed during gestation, lactation, pre-pubertal and pubertal period up to the age of 70 days, when they were sacrificed and analyzed. Animals exposed to the low dose were exposed to an environmentally relevant concentration (around 25 ng/kg bw) (Zinedine et al., 2007).

We did not observe any changes of the body weight or weight of reproductive organs between the control and experimental groups. However, we observed decreased sperm quality mainly in the group exposed to low concentration of ZEA. In this group we observed decreased sperm concentration, increased amount of morphologically abnormal spermatozoa and increased binding of apoptotic marker annexin V. Using the qPCR technique we evaluated the expression profile of 28 testicular genes. The tested genes can be divided into five groups according to their function – genes expressed in the germ cells (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). In general, we observed more alterations in expression of selected genes in animals exposed to the low dose of ZEA. In this group, we detected decreased expression of the Vegfa gene, which is specifically expressed in spermatogonial cells. We also detected decreased expression of several genes specific for spermatocytes – Sycp3, Ccna1, and Grth. Interestingly, in the group exposed to the higher dose of ZEA we did not observe any significant changes in the expression of the tested germ-cell specific genes. Regarding genes expressed in Sertoli cells, we noticed significantly decreased expression of the Sox9 gene in both groups and

also decreased expression of the Wt1 gene in the group exposed to the high dose. In the case of genes related to epigenetic processes, we observed decreased expression of genes for Ccnd1 and Dnmt1 in the animals exposed to the low dose of ZEA and decreased expression of genes for Ccnd1, Kdm4a and Spata2 in animals exposed to the high dose.

We have shown that ZEA is able to negatively influence the sperm quality, mainly sperm concentration and morphology. Based on our results from gene expression analysis we assume that the most affected cells are spermatogonia and meiotic germ cells. In this study, animals exposed to the low ZEA concentration were affected markedly more than animals exposed to the high ZEA concentration. A similar observation was obtained in our study with bisphenol A, where a lower dose had a greater effect on sperm quality and the number of births *in vivo* (Peknicova et al., 2002). This phenomenon is often observed in endocrinology. Hormones operate in quite low concentrations, commonly below their dissociation constant. At these low concentrations, the receptor saturation appears to be most sensitive to the ligand concentration (the relationship is almost linear), while at higher concentrations of the ligand (hormone), we cannot apply the linear relationship between the ligand concentration and receptor saturation. This phenomenon is called inverted U dose response or non-monotonic dose response (Welshons et al., 2003). 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 (Fox, 1975). Apparently different responses may therefore be observed depending on the hormone concentration. For example, proliferation of GH3 rat pituitary cells was observed at 0.001-0.01 nM concentration of 17β-estradiol, while at 0.1 nM concentration the hormone induced synthesis of prolactin (Amara and Dannies, 1983). It has also been shown that the same chemicals that stimulate growth of MCF7 cells at lower concentration can slow down MCF-7 growth at higher concentrations. These examples indicate that different hormone concentrations may lead to the activation of different genes and subsequently induce different phenotypes. Too high concentrations of certain hormones can even lead to cytotoxicity that is not dependent on the presence of the receptor (Welshons et al., 2003).

4.3. Expression of spermatogenesis-related genes in specimens from testicular biopsies of infertile men who underwent TESE for the ICSI procedure. (Research paper 3.4.)

Many couples in our population experience some form of infertility problems. Many of them search for medical assistance and use services of some of the centers of assisted reproduction. The causes of male infertility are often associated with lower sperm count – oligozoospermia, decreased motility – asthenozoospermia, morphology defects – teratozoospermia, or a combination of these defects. A very serious defect is the absence of sperm in the ejaculate – azoospermia. Azoospermic patients still have the opportunity to father a child with the help of testicular sperm extraction (TESE) and ICSI (Mansour, 1998). At present, the only reliable and routinely used predictor of successful TESE is testicular histology. In this study, we used specimens from testicular biopsies of azoospermic patients who underwent TESE and we analyzed expression of selected genes. We assumed that this analysis could provide an additional approach to increase the prediction of successful TESE.

Genes tested in this study were – GAPDH (expressed in somatic testicular cells and spermatogonia); MND1/GAJ and SPATA22 (expressed prior to meiotic division); GAPDHS and ACR (expressed in haploid spermatids). In total we analyzed 34 samples, out of which 9 were diagnosed as Sertoli cell only (SCO); 12 as maturation arrest at spermatocyte stage (MA); 12 as hypospermatogenesis with a few spermatozoa present (HS) and one as obstructive azoospermia. In three samples from the HS group and in six samples from the MA group we detected no or low expression of the studied genes. In one sample from the HS group and two samples from the MA group we noticed expression of MND1 and SPATA22, but we did not detect any ACR or GAPDHS gene products. In the SCO group, in two samples we observed decreased expression of the tested genes, whereas in the remaining seven samples we noticed only residual presence of GAPDHS, ACR and SPATA22.

In this study, the expression analysis of selected genes provided sensitive confirmation of histologically diagnosed SCO. Concerning MA and hypospermatogenesis, gene expression analysis could help determine in which stage the spermatogenesis arrest occurred and thus help assess the most suitable procedure for each patient.

5. Conclusion

In the presented study, we focused on the effect of two endocrine disruptors (TBBPA and ZEA) on various male reproductive parameters using a mouse model. According to our results, these pollutants have a significant effect on spermatogenesis and can negatively influence the sperm quality. In the case of TBBPA we even observed a slight transgenerational effect which has been previously observed in some other endocrine disruptors (Anway and Skinner, 2008). In the case of ZEA, we observed a negative effect mainly on sperm parameters and expression of important testicular genes. We also noticed that the lower concentration of ZEA had a stronger effect than the high concentration. Many toxicological studies use high concentrations of the tested compounds and it is assumed that if high concentrations do not have any negative effect, the same is true for the low concentrations. Our as well as other studies have disproved this hypothesis, especially for compounds that can be hormonally active.

Nevertheless, during our experiments we did not observe any significant effect on fertility of the exposed animals. However, we have to realize that humans and wildlife are exposed to thousands of various organic or synthetic compounds which can influence their health and/or fertility. With growing number of infertile couples and rapidly decreasing sperm quality reported from European and American countries, it is crucial to reveal compounds that are hazardous for our reproductive health and to eliminate their usage.

The growing number of infertile couples is linked with higher demand for assisted reproductive technology (ART). At present, many techniques are available and it is important to choose the proper one for each patient. For this purpose there are diagnostic methods that help select the most suitable ART. In our study, we have shown that gene expression analysis could be an important additional tool for more precise and help to choose the best approach for each patient.

6. References

Abou-Haila A, Tulsiani DR. 2000. Mammalian sperm acrosome: formation, contents, and function. Arch Biochem Biophys 379:173-182.

Ahmed EA, Barten-van Rijbroek AD, Kal HB, Sadri-Ardekani H, Mizrak SC, van Pelt AM, de Rooij DG. 2009. Proliferative activity in vitro and DNA repair indicate that adult mouse and human Sertoli cells are not terminally differentiated, quiescent cells. Biol Reprod 80:1084-1091.

Alaee M, Arias P, Sjodin A, Bergman A. 2003. An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. Environ Int 29:683-689.

Allen MJ, Lee C, Lee JDt, Pogany GC, Balooch M, Siekhaus WJ, Balhorn R. 1993. Atomic force microscopy of mammalian sperm chromatin. Chromosoma 102:623-630.

Alm H, Greising T, Brussow KP, Torner H, Tiemann U. 2002. The influence of the mycotoxins deoxynivalenol and zearalenol on in vitro maturation of pig oocytes and in vitro culture of pig zygotes. Toxicol In Vitro 16:643-648.

Amara JF, Dannies PS. 1983. 17 beta-Estradiol has a biphasic effect on gh cell growth. Endocrinology 112:1141-1143.

Anway MD, Skinner MK. 2008. Transgenerational effects of the endocrine disruptor vinclozolin on the prostate transcriptome and adult onset disease. Prostate 68:517-529.

Aoki VW, Liu L, Carrell DT. 2005. Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. Hum Reprod 20:1298-1306.

Balhorn R. 2007. The protamine family of sperm nuclear proteins. Genome Biol 8:227.

Benzoni E, Minervini F, Giannoccaro A, Fornelli F, Vigo D, Visconti A. 2008. Influence of in vitro exposure to mycotoxin zearalenone and its derivatives on swine sperm quality. Reprod Toxicol 25:461-467.

Birnbaum LS, Staskal DF. 2004. Brominated flame retardants: cause for concern? Environ Health Perspect 112:9-17.

Bisig CG, Guiraldelli MF, Kouznetsova A, Scherthan H, Hoog C, Dawson DS, Pezza RJ. 2012. Synaptonemal complex components persist at centromeres and are required for homologous centromere pairing in mouse spermatocytes. PLoS Genet 8:e1002701.

Bremner WJ, Millar MR, Sharpe RM, Saunders PT. 1994. Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. Endocrinology 135:1227-1234.

Brunner AM, Nanni P, Mansuy IM. 2014. Epigenetic marking of sperm by post-translational modification of histones and protamines. Epigenetics Chromatin 7:2.

Brykczynska U, Hisano M, Erkek S, Ramos L, Oakeley EJ, Roloff TC, Beisel C, Schubeler D, Stadler MB, Peters AH. 2010. Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. Nat Struct Mol Biol 17:679-687.

Cano-Sancho G, Sanchis V, Ramos AJ, Marin S. 2013. Effect of food processing on exposure assessment studies with mycotoxins. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 30:867-875.

Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. 1992. Evidence for decreasing quality of semen during past 50 years. BMJ 305:609-613.

Carrell DT, Emery BR, Hammoud S. 2007. Altered protamine expression and diminished spermatogenesis: what is the link? Hum Reprod Update 13:313-327.

Carrell DT, Liu L. 2001. Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. J Androl 22:604-610.

Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, Eddy EM. 2001. Haploinsufficiency of protamine-1 or -2 causes infertility in mice. Nat Genet 28:82-86.

Corzett M, Mazrimas J, Balhorn R. 2002. Protamine 1: protamine 2 stoichiometry in the sperm of eutherian mammals. Mol Reprod Dev 61:519-527.
Crisp TM, Clegg ED, Cooper RL, Wood WP, Anderson DG, Baetcke KP, Hoffmann JL, Morrow MS, Rodier DJ, Schaeffer JE, Touart LW, Zeeman MG, Patel YM. 1998. Environmental endocrine disruption: an effects assessment and analysis. Environ Health Perspect 106 Suppl 1:11-56.
Damstra T, Page SW, Herrman JL, Meredith T. 2002. Persistent organic pollutants: potential health effects? J Epidemiol Community Health 56:824-825.
de Kretser DM, Loveland KL, Meinhardt A, Simorangkir D, Wreford N. 1998. Spermatogenesis. Hum Reprod 13 Suppl 1:1-8.
Dym M. 1994. Spermatogonial stem cells of the testis. Proc Natl Acad Sci U S A 91:11287-11289.
Dym M, Fawcett DW. 1971. Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. Biol Reprod 4:195-215.
Eddy EM. 2002. Male germ cell gene expression. Recent Prog Horm Res 57:103-128.
Eddy EM, O'Brien DA. 1998. Gene expression during mammalian meiosis. Curr Top Dev Biol 37:141-200.
Elzeinova F, Novakova V, Buckiova D, Kubatova A, Peknicova J. 2008. Effect of low dose of vinclozolin on reproductive tract development and sperm parameters in CD1 outbred mice. Reprod Toxicol 26:231-238.
Fernandez MP, Ikonomou MG, Buchanan I. 2007. An assessment of estrogenic organic contaminants in Canadian wastewaters. Sci Total Environ 373:250-269.
Filipiak E, Walczak-Jedrzejowska R, Oszukowska E, Guminska A, Marchlewska K, Kula K, Slowikowska-Hilczer J. 2009. Xenoestrogens diethylstilbestrol and zearalenone negatively influence pubertal rat's testis. Folia Histochem Cytobiol 47:S113-120.
Fini JB, Le Mevel S, Turque N, Palmier K, Zalko D, Cravedi JP, Demeneix BA. 2007. An in vivo multiwell-based fluorescent screen for monitoring vertebrate thyroid hormone disruption. Environ Sci Technol 41:5908-5914.
Fox TO. 1975. Androgen- and estrogen-binding macromolecules in developing mouse brain: biochemical and genetic evidence. Proc Natl Acad Sci U S A 72:4303-4307.
Fremont L. 2000. Biological effects of resveratrol. Life Sci 66:663-673.
Govin J, Caron C, Lestrat C, Rousseaux S, Khochbin S. 2004. The role of histones in chromatin remodelling during mammalian spermiogenesis. Eur J Biochem 271:3459-3469.
Hammoud SS, Nix DA, Hammoud AO, Gibson M, Cairns BR, Carrell DT. 2011. Genome-wide analysis identifies changes in histone retention and epigenetic modifications at developmental and imprinted gene loci in the sperm of infertile men. Hum Reprod 26:2558-2569.
Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. 2009. Distinctive chromatin in human sperm packages genes for embryo development. Nature 460:473-478.
Hirsh A. 2003. Male subfertility. BMJ 327:669-672.
Jamsai D, O'Bryan MK. 2011. Mouse models in male fertility research. Asian J Androl 13:139-151.
Jodar M, Oliva R. 2014. Protamine alterations in human spermatozoa. Adv Exp Med Biol 791:83-102.
Khara KK, Vlad M, Griffiths M, Kennedy CR. 1997. Human protamines and male infertility. J Assist Reprod Genet 14:282-290.
Kiani J, Grandjean V, Liebers R, Tuorto F, Ghanbarian H, Lyko F, Cuzin F, Rassoulzadegan M. 2013. RNA-mediated epigenetic heredity requires the cytosine methyltransferase Dnmt2. PLoS Genet 9:e1003498.
Kierkegaard A, Sellstrom U, McLachlan MS. 2009. Environmental analysis of higher brominated diphenyl ethers and decabromodiphenyl ethane. J Chromatogr A 1216:364-375.
Kim IH, Son HY, Cho SW, Ha CS, Kang BH. 2003. Zearalenone induces male germ cell apoptosis in rats. Toxicol Lett 138:185-192.

Kitamura S, Jinno N, Ohta S, Kuroki H, Fujimoto N. 2002. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. Biochem Biophys Res Commun 293:554-559.

Korner W, Hanf V, Schuller W, Bartsch H, Zwirner M, Hagenmaier H. 1998. Validation and application of a rapid in vitro assay for assessing the estrogenic potency of halogenated phenolic chemicals. Chemosphere 37:2395-2407.

Kuiper-Goodman T, Scott PM, Watanabe H. 1987. Risk assessment of the mycotoxin zearalenone. Regul Toxicol Pharmacol 7:253-306.

Kupka MS, Ferraretti AP, de Mouzon J, Erb K, D'Hooghe T, Castilla JA, Calhaz-Jorge C, De Geyter C, Goossens V, European Ivf-Monitoring Consortium ftESoHR, Embryology. 2014. Assisted reproductive technology in Europe, 2010: results generated from European registers by ESHREdagger. Hum Reprod 29:2099-2113.

Kyselova V, Peknicova J, Boubelik M, Buckiova D. 2004. Body and organ weight, sperm acrosomal status and reproduction after genistein and diethylstilbestrol treatment of CD1 mice in a multigenerational study. Theriogenology 61:1307-1325.

Legler J. 2008. New insights into the endocrine disrupting effects of brominated flame retardants. Chemosphere 73:216-222.

Li Y, Lalancette C, Miller D, Krawetz SA. 2008. Characterization of nucleohistone and nucleoprotamine components in the mature human sperm nucleus. Asian J Androl 10:535-541.

Liebers R, Rassoulzadegan M, Lyko F. 2014. Epigenetic regulation by heritable RNA. PLoS Genet 10:e1004296.

Lui WY, Cheng CY. 2012. Transcriptional regulation of cell adhesion at the blood-testis barrier and spermatogenesis in the testis. Adv Exp Med Biol 763:281-294.

Mansour R. 1998. Intracytoplasmic sperm injection: a state of the art technique. Hum Reprod Update 4:43-56.

Marshall GR, Zorub DS, Plant TM. 1995. Follicle-stimulating hormone amplifies the population of differentiated spermatogonia in the hypophysectomized testosterone-replaced adult rhesus monkey (Macaca mulatta). Endocrinology 136:3504-3511.

Meerts IA, van Zanden JJ, Luijks EA, van Leeuwen-Bol I, Marsh G, Jakobsson E, Bergman A, Brouwer A. 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. Toxicol Sci 56:95-104.

Mengual L, Ballesca JL, Ascaso C, Oliva R. 2003. Marked differences in protamine content and P1/P2 ratios in sperm cells from percoll fractions between patients and controls. J Androl 24:438-447.

Minervini F, Giannoccaro A, Cavallini A, Visconti A. 2005. Investigations on cellular proliferation induced by zearalenone and its derivatives in relation to the estrogenic parameters. Toxicol Lett 159:272-283.

O'Donnell L, McLachlan RI, Wreford NG, de Kretser DM, Robertson DM. 1996. Testosterone withdrawal promotes stage-specific detachment of round spermatids from the rat seminiferous epithelium. Biol Reprod 55:895-901.

Ogunbayo OA, Lai PF, Connolly TJ, Michelangeli F. 2008. Tetrabromobisphenol A (TBBPA), induces cell death in TM4 Sertoli cells by modulating Ca2+ transport proteins and causing dysregulation of Ca2+ homeostasis. Toxicol In Vitro 22:943-952.

Oliva R. 2006. Protamines and male infertility. Hum Reprod Update 12:417-435.

Oliva R, Bazett-Jones D, Mezquita C, Dixon GH. 1987. Factors affecting nucleosome disassembly by protamines in vitro. Histone hyperacetylation and chromatin structure, time dependence, and the size of the sperm nuclear proteins. J Biol Chem 262:17016-17025.

Peknicova J, Kyselova V, Buckiova D, Boubelik M. 2002. Effect of an endocrine disruptor on mammalian fertility. Application of monoclonal antibodies against sperm proteins as markers for testing sperm damage. Am J Reprod Immunol 47:311-318.

Poongothai J, Gopenath TS, Manonayaki S. 2009. Genetics of human male infertility. Singapore Med J 50:336-347.
Prelusky DB, Scott PM, Trenholm HL, Lawrence GA. 1990. Minimal transmission of zearalenone to milk of dairy cows. J Environ Sci Health B 25:87-103.
Roy JR, Chakraborty S, Chakraborty TR. 2009. Estrogen-like endocrine disrupting chemicals affecting puberty in humans--a review. Med Sci Monit 15:RA137-145.
Ruhr LP, Osweiler GD, Foley CW. 1983. Effect of the estrogenic mycotoxin zearalenone on reproductive potential in the boar. Am J Vet Res 44:483-485.
Samuelsen M, Olsen C, Holme JA, Meussen-Elholm E, Bergmann A, Hongslo JK. 2001. Estrogen-like properties of brominated analogs of bisphenol A in the MCF-7 human breast cancer cell line. Cell Biol Toxicol 17:139-151.
Sharpe RM, McKinnell C, Kivlin C, Fisher JS. 2003. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction 125:769-784.
Simone LC, Georgesen CJ, Simone PD, Wang X, Solheim JC. 2012. Productive association between MHC class I and tapasin requires the tapasin transmembrane/cytosolic region and the tapasin C-terminal Ig-like domain. Mol Immunol 49:628-639.
Sjodin A, Carlsson H, Thuresson K, Sjolin S, Bergman A, Ostman C. 2001. Flame retardants in indoor air at an electronics recycling plant and at other work environments. Environ Sci Technol 35:448-454.
Smith LB, Walker WH. 2014. The regulation of spermatogenesis by androgens. Semin Cell Dev Biol 30:2-13.
Sofikitis N, Giotitsas N, Tsounapi P, Baltogiannis D, Giannakis D, Pardalidis N. 2008. Hormonal regulation of spermatogenesis and spermiogenesis. J Steroid Biochem Mol Biol 109:323-330.
Sonnack V, Failing K, Bergmann M, Steger K. 2002. Expression of hyperacetylated histone H4 during normal and impaired human spermatogenesis. Andrologia 34:384-390.
Steger K. 1999. Transcriptional and translational regulation of gene expression in haploid spermatids. Anat Embryol (Berl) 199:471-487.
Stewart MN, Dawson DS. 2008. Changing partners: moving from non-homologous to homologous centromere pairing in meiosis. Trends Genet 24:564-573.
Stouder C, Paoloni-Giacobino A. 2011. Specific transgenerational imprinting effects of the endocrine disruptor methoxychlor on male gametes. Reproduction 141:207-216.
Suarez-Quian CA, Martinez-Garcia F, Nistal M, Regadera J. 1999. Androgen receptor distribution in adult human testis. J Clin Endocrinol Metab 84:350-358.
Swan SH, Elkin EP, Fenster L. 1997. Have sperm densities declined? A reanalysis of global trend data. Environ Health Perspect 105:1228-1232.
Swan SH, Elkin EP, Fenster L. 2000. The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996. Environ Health Perspect 108:961-966.
Takigami H, Suzuki G, Hirai Y, Sakai S. 2008. Transfer of brominated flame retardants from components into dust inside television cabinets. Chemosphere 73:161-169.
Takigami H, Suzuki G, Hirai Y, Sakai S. 2009. Brominated flame retardants and other polyhalogenated compounds in indoor air and dust from two houses in Japan. Chemosphere 76:270-277.
Tarulli GA, Stanton PG, Meachem SJ. 2012. Is the adult Sertoli cell terminally differentiated? Biol Reprod 87:13, 11-11.
Tsakmakidis IA, Lymberopoulos AG, Alexopoulos C, Boscos CM, Kyriakis SC. 2006. In vitro effect of zearalenone and alpha-zearalenol on boar sperm characteristics and acrosome reaction. Reprod Domest Anim 41:394-401.

van der Heijden GW, Ramos L, Baart EB, van den Berg IM, Derijck AA, van der Vlag J, Martini E, de Boer P. 2008. Sperm-derived histones contribute to zygotic chromatin in humans. BMC Dev Biol 8:34.

Van der Ven LT, Van de Kuil T, Verhoef A, Verwer CM, Lilienthal H, Leonards PE, Schauer UM, Canton RF, Litens S, De Jong FH, Visser TJ, Dekant W, Stern N, Hakansson H, Slob W, Van den Berg M, Vos JG, Piersma AH. 2008. Endocrine effects of tetrabromobisphenol-A (TBBPA) in Wistar rats as tested in a one-generation reproduction study and a subacute toxicity study. Toxicology 245:76-89.

Walker WH. 2011. Testosterone signaling and the regulation of spermatogenesis. Spermatogenesis 1:116-120.

Wang RS, Yeh S, Tzeng CR, Chang C. 2009. Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocr Rev 30:119-132.

Watanabe I, Kashimoto T, Tatsukawa R. 1983. Identification of the flame retardant tetrabromobisphenol-A in the river sediment and the mussel collected in Osaka. Bull Environ Contam Toxicol 31:48-52.

Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS. 2003. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ Health Perspect 111:994-1006.

Willems A, Batlouni SR, Esnal A, Swinnen JV, Saunders PT, Sharpe RM, Franca LR, De Gendt K, Verhoeven G. 2010. Selective ablation of the androgen receptor in mouse sertoli cells affects sertoli cell maturation, barrier formation and cytoskeletal development. PLoS One 5:e14168.

Wittassek M, Angerer J. 2008. Phthalates: metabolism and exposure. Int J Androl 31:131-138.

Wosnitzer MS, Goldstein M. 2014. Obstructive azoospermia. Urol Clin North Am 41:83-95.

Yang JY, Wang GX, Liu JL, Fan JJ, Cui S. 2007a. Toxic effects of zearalenone and its derivatives alpha-zearalenol on male reproductive system in mice. Reprod Toxicol 24:381-387.

Yang JY, Zhang YF, Wang YQ, Cui S. 2007b. Toxic effects of zearalenone and alpha-zearalenol on the regulation of steroidogenesis and testosterone production in mouse Leydig cells. Toxicology in Vitro 21:558-565.

Zhao M, Shirley CR, Hayashi S, Marcon L, Mohapatra B, Suganuma R, Behringer RR, Boissonneault G, Yanagimachi R, Meistrich ML. 2004. Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. Genesis 38:200-213.

Zinedine A, Soriano JM, Molto JC, Manes J. 2007. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. Food Chem Toxicol 45:1-18.