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Influence of bisphenol S on quality of mouse oocytes and embryos

Diploma Thesis

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Declaration

I hereby declare that I worked on my thesis “Influence of bisphenol S on quality of mouse oocytes and embryos” independently, using scientific literature and sources listed in the Bibliography section.

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Vliv bisfenolu S na kvalitu myších oocytů a embryí

Souhrn:

Negativní účinky endokrinního disruptoru bisfenolu A (BPA) na savčí organismus byly již popsány a výsledkem jsou dnes mimo jiné i BPA-free plasty bez přídavku této látky. V praxi byl ale BPA nahrazen svým strukturním analogem bisfenolem S (BPS), jehož účinky na savčí reprodukci nejsou dosud přesně definovány. Cílem této práce bylo ověřit hypotézu, že *in vivo* expozice velmi nízkým dávkám BPS postihuje negenomické a epigenetické kvalitativní vlastnosti myších oocytů, dvoubuněčných embryí a časných blastocyst. Pětitýdenní samice myší kmene ICR byly po čtyři týdny vystaveny čtyřem různým koncentracím BPS (0; 0.001; 0.1; 10; 100 ng . g ž. hm.$^{-1}$. den^{-1}). Oocyty byly izolovány po hormonální stimulaci eCG a hCG, embrya byla vypláchnuta z hormonálně stimulovaných myší 1,5 a 4,5 dne po přirozeném zapuštění. K visualizaci α-tubulinu meiotického vřetene, pericentrinu, 5-metylcytosinu a specifických histonových modifikací bylo použito imunocytochemické barvení a konfokální mikroskop se spinning diskem (Olympus, Německo). Intenzita fluorescenčního signálu byla kvantifikována v programu pro analýzu obrazu (ImageJ, NIH). Výsledky prokázaly významné postižení struktury meiotického vřetene a změněné hodnoty DNA metylace u oocytů i embryí. Hodnoty metylace histonu H3K4 (H3K4me2) v MII oocytech, histonu H3K9 (H3K9me3) ve dvoubuněčných embryích a množství heterochromatin proteinu 1 (HP1) v blastocystách se významně lišily od kontrolní skupiny. Změny byly nejvýraznější po expozici velmi nízkou dávkou BPS (0.1 ng . g ž. hm.$^{-1}$. den^{-1}) a ve výsledcích se zároveň projevovala nelineární závislost na použité koncentraci. Koncentrace BPS relevantní ke koncentracím nacházejícím se v životním prostředí tedy představují riziko pro reprodukci samic a jejich potomků prostřednictvím epigenetického transgeneračního efektu. Jakékoli další použití nebo nahrazení analogů BPA by mělo být kriticky zhodnoceno v zájmu celosvětového lidského zdraví i životního prostředí, do kterého se tyto chemické látky uvolňují.

Klíčová slova: bisfenol S, oocyt, embryo, fertilizace, embryonální vývoj, epigenetika

Influence of bisphenol S on quality of mouse oocytes and embryos

Summary:

Well-known endocrine disruptor (ED) bisphenol A (BPA) and its adverse effects were already described and as a result, a lot of plastics are now BPA-free. However, BPA has been replaced with its analogue bisphenol S (BPS), and its effects on reproduction and development are not fully known yet. The aim of the thesis was to test the hypothesis that *in vivo* exposure to low doses of BPS affects non-genomic and epigenetic markers of murine oocytes, 2-cell embryos, and early blastocysts. 5-week-old female ICR mice were administered with four different BPS concentrations (0; 0.001; 0.1; 10; 100 ng . g bw^{-1} . day^{-1}) in 4-week exposure window. Oocytes were isolated after hormonal stimulation with eCG and hCG. Alternatively, hormonally stimulated mice were mated and 2-cell embryos/early blastocysts were obtained 1,5/4,5 days after mating, respectively. Oocytes and embryos were subjected to immunocytochemistry staining and imaging on confocal microscope with spinning disc (Olympus, Germany) for visualization of spindle α-tubulin, pericentrin, 5-methylcytosine and specific histone modifications. Fluorescence signal intensity was quantified and assessed in ImageJ software (NIH, USA). The results showed significant affection of meiotic spindle structure as well as altered genome-wide methylation levels in both oocytes and embryos. Methylation levels of histone H3K4 (H3K4me2) in MII oocytes and H3K9 (H3K9me3) in 2-cell embryos, as well as heterochromatin protein 1 (HP1) amount in blastocysts, varied significantly from the control group. All of these effects were the most profound with BPS dose as low as 0.1 ng . g bw^{-1} . day^{-1} and manifested in non-linear dose-response manner. Results suggest that the environmentally relevant doses of BPS pose a risk for female reproduction and their offspring through epigenetic-driven transgenerational effect. Further usage of BPA analogues should be critically assessed in concern of global human health and environmental pollution.

Keywords: bisphenol S, oocyte, embryo, fertilization, embryonic development, epigenetics

Content

1 Introduction

Oocyte maturation, fertilization, and early embryonic development are essential and susceptible hallmarks of the mammalian reproductive process. Successful progression through these periods depends on a myriad of signaling cascades, and any change in their balance can have severe consequences for the reproductive fitness of an individual, or his health in general when affected as an embryo. With the advent of epigenetics, there are obvious impacts that apply not only to the directly affected ones but also for their offspring through the transgenerational inheritance.

There are many environmental pollutants and ubiquitously present compounds currently that can interfere with mammalian reproduction. These so-called endocrine disruptors can act silently, over a long time-course and in low doses, resulting in impaired physiological processes in animals and humans. Bisphenols are indeed the ones man should be concerned about.

Bisphenol A (BPA) is already well known to have negative effects on human reproduction and fertility, and therefore its usage was largely abandoned, especially in products for children. However, its properties favorable for plastic goods were replaced with bisphenol S (BPS), an analogue of BPA. Some of BPS adverse effects have been already described, but there is still an urgent need to prove those with more studies. This thesis is focused on BPS effect on non-genomic and epigenetic factors, both essential for correct oocyte maturation, fertilization and further early embryonic development. Results can support a reevaluation of human health risks associated with bisphenols exposure and their replacement with structural analogues.

2 Scientific hypothesis and objectives of work

The aim of this work was to evaluate the effect bisphenol S poses for murine oocytes and embryos.

Following hypotheses were tested:

i) Low doses of bisphenol S affect meiotic spindle structure and chromosomal alignment in MII oocytes

ii) Bisphenol S affects the epigenetic pattern of murine oocytes and early embryos.

3 Literature overview

3.1 Oogenesis and folliculogenesis

To achieve successful reproduction, it is essential to produce fertilizable gametes. The important thing to emphasize is that the two essential processes, oogenesis and folliculogenesis, are inseparable from each other. Once the primordial follicle is activated, it goes along with the oocyte growth phase until it becomes sensitive to gonadotropins. After that, oocyte begins its pre-maturation phase, signaling the onset of the gonadotropin-dependent phase of folliculogenesis. These interactions between follicles and oocytes are mainly maintained with paracrine and gap junction types of signaling pathways and any deviation from their coordination and integration represents a failure in reproductive fitness of given species (Albertini, 2014).

3.1.1 Folliculogenesis and its regulation

Folliculogenesis is a highly regulated process during which somatic granulosa cells differentiate and oocytes are able to grow, mature and can be ovulated at the right time (Pangas and Rajkovic, 2015). Follicles and its somatic cells provide a specialized environment, nutrients, and signals for oocyte development (Voronina and Wessel, 2003). Female mice follicles start to develop in the early prenatal period, beginning with germ cell clusters (GCCs), each of which contains about eight or more germ cells. Germ cells in a cluster are connected via intercellular bridges and form syncytia (Pangas and Rajkovic, 2015). These bridges are a result of incomplete cytokinesis of mitotically dividing germ cells (Pepling and Spradling, 2001) as well as a result of their aggregation (Mork et al., 2012).

Murine germ cells, derived from the proximal epiblast and identified by expression of BLIMP1, PRDM1 and PRDM14 proteins, migrate through the primitive streak into the adjacent posterior embryonic endoderm, extraembryonic endoderm and allantois. This migration starts around the embryonic day E7.5. During the migratory phase, extensive genome reprogramming, such as DNA methylation, histone modification

and erasure of gene imprinting takes place in primordial germ cells (PGCs). These events may be essential for restoring totipotency to these cells (McCarthy and Rissman, 2014; Nikolic et al., 2016). In the later stages of gastrulation, the migration continues to the urogenital ridges, germ cells associate clusters and further proliferate up to 6,000 – 26,000 cells, depending on the mouse strain. At E13.5, these mitotically dividing oogonia enter meiosis and progress through prophase I to diplotene before arresting their cell cycle (Epifano and Dean, 2002). While some oocytes are arrested in pachytene and early diplotene and some in late diplotene and dictyate at birth, they will resume meiosis just before ovulation (Pangas and Rajkovic, 2015).

Somatic cells, that include granulosa and theca cells, are formed in genital ridges that arise from ventrolateral mesonephros and are bordered by coelomic epithelium. Syncytia of germ cells then aggregates with somatic cells precursors to form sex cords, later evolving in ovarian follicles (Epifano a Dean, 2002).

Formation of primordial follicles from GCC arises in mice around E17.5 through projections of somatic cells that grow among germ cells, disrupt intercellular bridges and individual oocytes are eventually enclosed with flat, squamous epithelial-derived somatic cells, called pre-granulosa cells. The total number of oocytes declines dramatically as nearly two thirds of them undergo cell death, ensuring that only germ cells that acquired functional mitochondria can form primordial follicles (Pepling and Spradling, 2001). This process of GCC breakdown is finished in postnatal day four. (Pangas and Rajkovic, 2015).

Mechanisms that regulate primordial follicle formation include programmed cell death regulators, growth factors and other signaling pathways (*e.g.* TGFß family members), transcription factors, steroid hormones, meiotic progression, and changes in cell adhesion (Pepling, 2012). Last but not least, oocyte itself plays a crucial role in follicle development, as shown in FIGα (an oocyte-specific, basic helix-loop-helix transcription factor) knockout mice with shrunken ovaries and no primordial follicles formed (Soyal et al., 2000).

Once formed, the amount of primordial follicles is thought to be the final pool of follicles for the whole reproductive lifespan of the female, according to the classical

dogma of reproductive biology. However, recent findings suggest that these dynamics do not have to be quite right. According to a pilot study of Johnson et al. (2004), there are germline stem cells present in the ovary that can generate new oocytes and new follicles can be formed even postnatally. They support their theory with findings of big proportions of atretic follicles, that would deplete the entire oocytes pool in a few weeks, as well as with evidence of newly formed primordial follicles after treatment with germ cell toxicant, busulphan. They also observed newly formed follicles with GFP-negative granulosa cells containing GFP-positive oocytes after introducing ovarian graft from GFP-negative mice into GFP-positive sibling. The more recent study suggests there are precursors of germ cells present in peripheral blood or bone marrow of mice (Tilly et al., 2009), and mitotically-active germ cells were isolated lately from women in reproductive age (White et al., 2012). Despite numbers of studies confirming these findings, the idea of germline stem cells still remains controversial (Pan et al., 2016).

In mice, the transition from inactive and quiescent primordial follicles to active primary follicles is set up by postnatal day 3 and primary follicles are characterized by cuboidal granulosa cells and growth of the oocyte beyond 20 μm (Pangas and Rajkovic, 2015). This event is also called initial recruitment, a continuous process that starts just after follicle formation and long before pubertal onset. Both decrease of inhibitory influences and increase of stimulatory factors are involved in follicle growth initiation, but the process remains largely gonadotropin-independent (McGee and Hsueh, 2000). Initiation of follicular growth and progression beyond the primary follicle stage appear to involve interactions between KIT, a tyrosine kinase receptor found in oocytes, and KITL (KIT ligand, encoded by *Kitl*), either secreted from or on the surface of granulosa cells. The disruption of receptor–ligand communications between somatic cells and the central germ cell might affect the production of paracrine factors necessary for follicular growth (Epifano and Dean, 2002). Growth factors of TGFß family, especially GDF9 and BMP15 expressed in oocyte under control of NOBOX are involved as well. Mice lacking NOBOX cannot form growing follicles, have an arrest in folliculogenesis at the transition from primordial to primary follicles, and are sterile (Rajkovic, 2004). Anti-Müllerian hormone (AMH) seems to regulate initial recruitment by decreasing the number of follicles recruited, and there is an earlier primordial follicle pool depletion in wild-type (WT) mice in contrast

to AMH knockout mice (Durlinger, 1999). *Sohlh1*, *Sohlh2* and *Lhx8* genes coding for oocyte-specific basic helix-loop-helix transcription factors also appear to be crucial in maintaining the transition, and mice ovaries are completely devoid of follicles by 3 weeks of age in their absence (Pangas et al., 2006; Choi et al., 2008).

After initial recruitment, secondary follicles are distinguished from primary ones as having more than one layer of granulosa cells as well as acquiring an additional somatic cell layer. This thecal cell layer begins to form in growing follicles around the basement membrane and ultimately differentiates into the *theca interna* and *theca externa*. The *theca interna* layer differentiates into theca interstitial cells, whereas the *theca externa* layer differentiates into smooth muscle cells. *Theca* development also coincides with the development of numerous small blood vessels, presumably *via* angiogenesis. At postnatal day 14 ovary looks similar to a day 7 ovary but is larger and contains multiple secondary follicles. Follicles are also degenerating at this stage, as lack of appropriate pituitary FSH levels does not support further follicular development (Pangas and Rajkovic, 2015).

As the follicle progresses to the antral phase, it becomes sensitive to extraovarian stimulation by gonadotropins through the hypothalamic–pituitary–gonadal (HPG) axis. The antral follicle is distinguished by a cavity filled with interstitial fluid, formed as previously scattered areas of fluid among granulosa cells coalesce. Granulosa cells are also divided into two kinds at this time – the mural cells lining the basement membrane and the cumulus cells surrounding the oocyte, subsequently forming the *cumulus oophorus*. Essential for the preantral-to-antral transition are connexins, proteins that form intercellular junctions and maintain metabolites transport. (Pangas and Rajkovic, 2015).

After puberty onset, stimulation by cyclic gonadotropins allows the survival and continued growth of only a limited number of antral follicles that will reach the preovulatory stage. The decrease of both somatic and germ cells in the ovary is mediated by apoptosis. Although apoptosis can occur at all stages of follicle development, the transition from secondary (preantral) to antral follicle is the most susceptible to atresia in rodents.

A cohort of antral follicles with 0.2 – 0.4 mm in diameter is subjected to cyclic recruitment as the circulating FSH increases during each reproductive cycle (McGee and Hsueh, 2000). The pituitary glycoprotein FSH plays an essential role in reproduction through interaction with gonadal FSH receptors. FSH is a dimeric glycoprotein composed of a unique β subunit complexed with a common α subunit that is shared with the thyroid-stimulating hormone, LH, and chorionic gonadotropin. FSH binds to the FSH receptor and activates cell signals that lead to germ cell maturation and follicular growth. It does so by stimulating the mural granulosa cells to proliferate, aromatize androgens to estrogens and to express LH receptors (Pangas and Rajkovic, 2015). An important factor that inhibits FSH-stimulated follicle growth is AMH, which mode of action lies in diminishing the sensitivity of follicles for FSH and mice that do not express AMH have more pronounced antral follicle growth after FSH stimulation than WT mice (Durlinger et al., 2001).

With the greater size of follicles and more LH receptors present on them, LH stimulates thecal cell C19 androgens production, which are conversed in C18 estrogens with a cytochrome P450 enzyme aromatase. Follicle-derived estrogens have a positive feedback on both the hypothalamus and pituitary to trigger the gonadotropin surge of LH that precedes ovulation (Pangas and Rajkovic, 2015). The action of estrogen production is being amplified by locally produced IGF-I, activin and BMP-2, -4 and -7, but counteracted by epidermal growth factor (EGF) (Van Den Hurk and Zhao, 2005). FSH secretion from the pituitary is inhibited with inhibin secreted by granulosa cells of the largest follicles. Once the follicles through complicated interactions of the oocyte, granulosa and theca cells, pituitary and hypothalamus are selected, LH surge triggers the process of ovulation, the follicle wall is broken down and the cumulus-oocyte complex is released. Granulosa cells remaining in the post-ovulatory follicle undergo luteinization, during which cells acquire steroidogenic morphology and start to produce progesterone, essential for uterine preparation and maintenance of pregnancy. Follicle development ends with the formation of the corpus luteum. In mice, the entire process of the primordial follicle to maturity takes approximately 3 weeks (Pangas and Rajkovic, 2015).

3.1.2 Growing and maturation of the oocyte

As stated before, oocytes originate from PGCs transformed into oogonia in fetal ovary. Oogonia show a high frequency of mitotic divisions, ultimately ending up in meiosis and becoming oocytes. These oocytes pass through leptotene, zygotene and pachytene of prophase I, and finally arrest their cell cycle in dictyotene. That is how oocytes start the second phase of their development – the growth phase.

3.1.2.1 Growth

The growth phase of oogenesis relies heavily on direct forms of paracrine signaling between the oocyte and surrounding somatic cells without direct involvement of gonadotropins. The transition from a quiescent, non-growing state within the primordial follicle, to that of active growth, is one of the most dramatic examples of cellular hypertrophy known. Expansion in size occurs as a consequence of prominent gene activation, selective protein synthesis, and hyperplasia of organelles. The oocyte, arrested in diplotene of prophase I, exhibits a burst of transcriptional activity upon primordial follicle activation that persists until the oocyte has completed growth. Among the new mRNA and proteins synthesized are i) those required for housekeeping and sustaining hypertrophy, ii) those involved with regulation of the meiotic cell cycle, iii) those required for embryonic development. All these three classes represent so-called maternal effect genes. A common feature of growing oocytes is also organelle accumulation and their migration to the oocyte cortex, mainly involving mitochondria, ribosomes, Golgi complexes and cortical granules, all of them participating in fertilization process (Albertini, 2014).

One of the most striking changes during the growth phase of an oocyte is its secretion of a glycoprotein membrane by Golgi complexes, the *zona pellucida* (ZP), which forms a protective coat around the oocyte consisting of three glycoproteins, ZP1, ZP2, and ZP3. In contrast to ZP1 and ZP3, expression of ZP2 occurs already in primordial follicles. The zona proteins are essential for normal follicle development and their expression requires transcription factor FIGα. The granulosa cells not only become

coupled with each other but also form gap junctions on the oolemma with the oocyte *via* processes passing through the developing ZP. These junctional contacts are formed by connexin proteins of which connexin 37 is expressed by the oocytes at all stages of folliculogenesis. The gap junctions facilitate a two-directional communication and allow transfer of nutrients, metabolic precursors, informational molecules, inhibitory agents (such as cAMP) and stimulatory meiotic signals. Through the junctional communication or by direct cell-cell contact, the growing oocyte promotes the growth of the follicular cells, and *vice versa*, follicular granulosa cells ensure the growth of the oocyte enclosed in the follicle. (Van Den Hurk and Zhao, 2005).

Oocyte at this stage is typical with so-called germinal vesicle (GV) – a large nucleus containing prominent nucleoli. Oocytes can be classified according to the state of chromatin surrounding these nucleoli either as non-surrounded nucleolus (NSN) oocytes with more dispersed and defined chromatin around the nucleoli, or as surrounded nucleolus (SN) oocytes where chromatin is highly condensed and closely surrounds the nucleoli (Zuccotti et al., 2005; Albertini, 2014). This chromatin organization has also been found in humans (Parfenov et al., 1989). The state of chromatin (condensed heterochromatin or diffused euchromatin) varies with the level of transcription during oocyte growth, ultimately forming heterochromatin encapsulated nucleoli as the oocyte progresses to the end of the growth phase and transcription ceases. In rodents, among genes being essential for oocyte to transcript during the growth phase are previously stated *Bmp15*, *Gdf9*, *NoboX*, *Sohlh1*, *Sohlh2*, *Lhx8* and the transcriptional factor *Oct4* (Albertini, 2014). Expression of *Oct4* in transcriptionally active growing NSN oocytes seems to be in positive correlation with the capability of these oocytes to resume meiosis and be ovulated since low levels of *Oct4* transcripts are associated with apoptosis in PGCs as well. The expression also seems to be under control of gonadotropins, when *Oct4* is upregulated both after eCG and hCG administration in mice. This suggests a twofold role of OCT4 in the recruitment of oocytes for initiating growth and in the selection of oocytes for ovulation (Monti et al., 2006).

3.1.2.2 Pre-maturation phase

In pre-maturation phase, the cell cycle is still off, but oocyte has already acquired its meiotic competence during the growth phase. In the mouse, meiotic competence acquisition is strictly correlated with the size of the oocyte (Albertini, 2014), and the maximum size of 70 μm has been reached already in secondary follicle stage (Van Den Hurk and Zhao, 2005). Meiotic and developmental competence of mouse oocytes is also associated with the state of chromatin surrounding the nucleoli. As shown by Zuccotti et al. (1998, 2005), SN oocytes are more likely to finish the meiotic maturation and develop beyond the embryonic 2-cell stage and blastocyst, whereas NSN oocytes lack this potential. Oocyte makes the transition from the growth phase to pre-maturation phase being in the antral follicle, when the follicle becomes sensitive to gonadotropins, signaling the onset of the gonadotropin-dependent phase of folliculogenesis (Albertini, 2014).

3.1.2.3 Maturation

In vivo, resumption of meiosis is initiated by a preovulatory LH surge and only occurs in fully grown, meiotically competent oocytes from dominant (preovulatory) follicles. Shortly after the time of LH peak, gap junctions present in cumulus cells zonal projections are disrupted during cumulus expansion (Van Den Hurk and Zhao, 2005). The current view is that LH causes mitogen-activated protein kinase (MAPK)-dependent phosphorylation and disruption of the connexin 43 gap junctions coupling the mural with the cumulus granulosa cells, restricting the flow of meiosis-arresting cGMP into the oocyte through connexin 37 gap junctions (Kidder and Vanderhyden, 2010). The oocyte then undergoes series of marked changes until the time of ovulation. These changes take place in the nucleus with ongoing meiotic division and proceeding from dictyate of prophase I to metaphase II with the absence of S-phase, as well as in cytoplasm with redistribution of cell organelles, migration of mitochondria to a perinuclear position and accumulation of cortical granules along the oolemma. A visible manifestation of finishing the first meiosis is also the formation of the first polar body (Van Den Hurk and Zhao, 2005). Mouse oocytes can be maintained in the prophase

arrest in culture indefinitely when they are enclosed in their follicles. However, when the follicle cells are removed, mouse oocytes enter maturation spontaneously (Voronina and Wessel, 2003). Matured oocyte can be also termed “an egg”.

GV oocytes destined to resume maturation will undergo breakdown of the germinal vesicle (GVBD), mediated by M-phase promoting factor (MPF; also the maturation promoting factor) (Sathananthan et al., 2006). The G2-to-M phase cell cycle progression, which occurs at the time of GVBD, depends on the activation of MPF, a dimer comprised of a CDK1 kinase (also referred to as CDC2) and cyclin B subunits. MPF activation leads to a cascade of cellular phosphorylation through Ser/Thr kinase activity, driving chromosome condensation and dissolution of the nuclear envelope (Downs, 2010). Full chromosome condensation is also accompanied by a dramatic reorganization of the microtubule cytoskeleton and formation of the bipolar meiotic spindle. The meiotic spindle is involved in various aspects of meiotic maturation including positioning of the cell division plane during cytokinesis and ensuring accurate repartition of the genetic material during the two successive meiotic divisions. Microtubule assembly is governed by microtubule-organizing centers (MTOCs) (Dumont and Brunet, 2010). MTOCs contain the signature protein components γ-tubulin and pericentrin, and undergo cell cycle–specific alterations during meiotic progression from diplotene GV stage to metaphase II. Therefore, oocyte MTOCs represent a major maternal determinant of embryo quality (Albertini, 2014). The microtubule organization is most likely regulated by MAPK pathways, as suggests the association of MAPK with MTOCs (Fan and Sun, 2004). The activity of MAPK is described hereinafter.

Prior to the surge of LH at estrus, and in order to maintain dictyate arrest, MPF activation is prevented by high levels of cyclic adenosine monophosphate (cAMP) in the oocyte. Level of cAMP is kept high by both external and internal factors. External cAMP is provided by the surrounding granulosa cells via gap junctions. Internal stimuli are mediated by supplies of cyclic guanosine monophosphate (cGMP), which suppresses oocyte phosphodiesterase 3A (PDE3A), which would otherwise degrade cAMP (Cohen and Holloway, 2014). Although PDE3 hydrolyzes both cAMP and cGMP, the rate of hydrolysis is 10-fold greater for cAMP than for cGMP, whereas the affinity for cGMP

is significantly higher. Thus, cGMP behaves as a competitive inhibitor of cAMP hydrolysis by PDE3. In oocytes, PDE3A is the predominant cAMP hydrolyzing PDE (Azevedo et al., 2014). Elevated cAMP results in increased protein kinase A (PKA) levels that phosphorylate key kinases, WEE1 and MYT1. These kinases, in turn, phosphorylate MPF. Phosphorylated MPF is inactive and cannot induce meiotic resumption. PKA also phosphorylates (thus inactivates) CDC25B, the major phosphatase required to activate MPF. PKA/CDC25B and PKA/WEE1 pathways most likely work in a synergistic manner to maintain an inactive CDC2/cyclin B complex and regulate meiotic resumption of mouse oocytes (Zhang et al., 2008; Cohen and Holloway, 2014). MPF activity is therefore negatively controlled by cAMP through regulation of WEE1 and MYT1 kinases and CDC25B phosphatase by PKA (Downs, 2010). Upon LH stimulation, cAMP levels decline due to an effect of LH on the granulosa, MPF is activated and GVBD occurs (Cohen and Holloway, 2014). Pharmacological manipulations to increase cAMP levels or injection of PKA in mammalian oocytes isolated from their follicles invariably produce a blockade of meiotic resumption, and PDE inhibitors, such as 3-isobutyl-1-methylxanthine, also block spontaneous mammalian oocyte maturation (Zhang et al., 2008).

Another factor that plays a role in MPF activation and meiotic resumption is MAPK. It accelerates MPF activation and is needed for suppression of interphase (namely the S-phase) between two divisions of meiosis and subsequent arrest at the second metaphase of meiosis (Voronina and Wessel, 2003). In mice, MAPK activation is not required for the initial activation of MPF and does so only after GVBD. In other species, by contrast, MAPK is activated before GVBD and might stimulate GVBD (Abrieu et al., 2001). MAPK comprises a family of Ser/Thr kinases that are optimally activated upon phosphorylation of threonine and tyrosine residues. Two isoforms are expressed in mammalian oocytes: ERK1 and ERK2, that are actively engaged in meiotic regulation. Activity is controlled by the upstream kinase, MEK, which, in turn, is regulated further upstream in vertebrate oocytes by the MEK kinase, MOS, a product of the c-mos protooncogene (Downs, 2010). ERK1 and ERK2 are also activated by growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF), and in response to insulin (Cargnello and Roux, 2011).

The wide range of functions regulated by the MAPKs is mediated through phosphorylation of several substrates, including members of a family of protein kinases termed MAPK-activated protein kinases (MAPKAPKs). This family includes, among others, the p90 ribosomal S6 kinases (RSKs). MOS and RSK2 (an isoform of RSK) were shown to contribute to the control of the meiotic cell cycle and the G2-to-M transition by phosphorylation and inhibition of the MYT1-WEE1, thus promoting further the activation of MPF and progression through to anaphase I (Cargnello and Roux, 2011; Cohen and Holloway, 2014). There is no intervening S-phase before entry into meiosis II, and oocytes progress to metaphase II rapidly, only to arrest again under the influence of cytostatic factor (CSF). High CSF activity induces stabilization and reaccumulation of MPF again by inhibiting the action of the anaphase-promoting complex, APC/C, which would otherwise target MPF for proteasomal degradation via polyubiquitination of cyclin B (Cohen and Holloway, 2014).

The dynamics of MPF and MAPK between the two meiotic divisions are distinct. Whereas the activity of MPF declines during the MI-MII transition, the activity of MAPK remains the same as at the beginning (approximately 2 h after GVBD). Inactivation of MAPK takes place 6 – 8 hrs after oocyte activation in MII, either by parthenogenetic stimulation or (*in vitro*) fertilization. However, the decrease in MPF levels precedes the decrease in MAPK levels after egg activation. Inactivation of MPF is in this case mediated by an APC-dependent mechanism activated by intracellular concentration of Ca^{2+} ions that rise upon fertilization or parthenogenetic activation. This stimulus then releases oocytes from meiotic metaphase II arrest (Fan and Sun, 2004).

Ca^{2+} signal is important for the regulation of meiotic cell cycle in oocytes through another kinase active in the maturation process termed Ca^{2+}/Calmodulin-dependent kinase II (CaMKII), a ubiquitously expressed Ser/Thr protein kinase activated by Ca^{2+} and calmodulin. Calcium-dependent pathways are essential for gonadotropin-induced oocyte meiotic resumption of mouse oocytes and are also involved in the progression of meiosis beyond metaphase I (Fan et al., 2003). Also, the release from metaphase II arrest after fertilization is mediated by CaMKII, which in the presence of PLX1 kinase activity triggers rapid and complete degradation of M-phase cyclins and sister chromatid segregation (Abrieu et al., 2001). Free Ca^{2+} ions enter the oocyte either from extracellular

stores or from intracellular calcium stores that have been mobilized via ryanodine or inositol triphosphate receptors (Petr et al., 2002). CaMKII inhibition leads to the inability of emission of the first polar body and arrest in the MI stage, and suppression of activity during egg activation resulted in a reduction in the amount and activity of MAPK. These two kinases were colocalized on the meiotic spindle, suggesting their potential adjacent and mutual regulation (Fan et al., 2003).

In addition to all the regulation activity mentioned, there are also gaseous molecules with signal transduction ability involved in the oogenesis as well. These gasotransmitters comprise three major molecules: NO, H_2S and CO. Their significance is pointed out mainly because of their absence in *in vitro* maturation protocols, that leads to an imperfect imitation of complete maturation (Nevoral et al., 2016).

3.2 Fertilization and early embryonic development

Fertilization takes place in the oviductal ampulla and it is the process by which the haploid gametes, sperm and egg, unite to produce a genetically distinct individual. In order for fertilization to occur, sperm must attain a fertile state through the process of capacitation. This process begins when sperms are exposed to seminal fluids and lasts about one hour in mice, with sperms residing for this prolonged period in the female reproductive tract. As a consequence of capacitation, sperm exhibit at least three modifications in their behavior related to fertility: hyperactivation of flagellar motility, regulation of signal transduction pathways permitting chemotaxis to progesterone and specific odorants, and expression of the ability of acrosome to interact with the egg. (Florman and Fissore, 2014).

3.2.1 Fertilization process

The morphological consequence of the acrosome reaction (AR) is multiple fusions of outer acrosome membrane with sperm plasma membrane, the formation of hybrid membrane vesicles, exposure of the inner membrane and exocytosis of acrosome contents. The general notion is that acrosome-intact sperm cannot penetrate ZP and fuse

with the egg (Wassarman, 1999). However, two basic models are available for AR time-course in mammals.

The first one states that sperm undergoes AR during the penetration of the cumulus cell mass (Jin et al., 2011; Okabe, 2013). Expanded *cumulus oophorus* with extracellular matrix rich in hyaluronic acid is indeed important during fertilization (both *in vivo* and *in vitro*) by entrapping the sperms and guiding them to the oocyte (Van Soom et al., 2002), but the real trigger of AR in the cumulus remains to be determined (Jin et al., 2011).

The second model is consistent with sperm reacting after the interaction with ZP and ZP proteins, with AR inducer being the ZP3 protein. Immediately after fertilization, ZP3 and ZP2 are modified, such as they are no longer recognized by sperm, serving as a prevention of polyspermy (Wassarman, 1999; Litscher et al., 2009). In this theory, acrosome-intact sperm can penetrate the cumulus layer using the hyaluronidase activity of plasma membrane protein PH-20 (Lin et al., 1994). PH-20, as well as proacrosin exposed on the acrosome-reacted sperm play an important role in the continued binding and subsequent penetration of the acrosome-reacted sperm through the ZP matrix (Gupta, 2014).

After the sperm penetrates *zona pellucida* and gets into the perivitelline space, it further interacts with the oolemma and ultimately fuses with the oocyte. Sperm-egg fusion is a highly regulated event and specific protein domains must come into association during this process. Sperm acquires the ability to fuse by exposing receptors on its surface upon acrosomal reaction, with the essential immunoglobulin superfamily protein being the IZUMO1. As recently shown, male mice with homozygous KO gene for IZUMO1 are completely infertile by the inability of sperm to fuse with the egg (Inoue et al., 2005). Later on, IZUMO1 binding partner on the oocyte was discovered and named as Juno. Similarly, female mice KO for Juno gene are infertile due to sperm-egg fusion inability, and these findings qualify Juno-IZUMO1 as the first cell-surface receptor pair essential for gamete recognition in any organism (Bianchi et al., 2014). Tight adhesion of the two cell membranes seems to be established by dimerization of monomeric IZUMO1 after recruitment by Juno and transfer to an unidentified receptor (Inoue et al., 2015). Juno seems to be also involved in the block to polyspermy, as it becomes

undetectable after fertilization in a time period that is in close agreement with membrane sperm-blocking timing, and is shed from the oolemma as vesicles (Bianchi and Wright, 2014). In addition to Juno-IZUMO1 interaction, there are other protein partners playing an important role in sperm-egg binding, such as integrin $\alpha_6\beta_1$ and a tetraspan membrane protein CD9 on the oolemma, and fertilin and cysteine-rich secretory protein-1 on the sperm membrane (Gupta, 2014).

Successful gamete fusion represents an activation signal for the egg arrested in metaphase II. The early event upon cell fusion is the calcium-dependent cortical reaction that prevents any other sperm to penetrate the egg (block to polyspermy). This event is mediated by G protein signal-transducing cascade, in which IP_3 induces the increase of intracellular calcium. During the cortical reaction, cortical granules located in the cortex of unfertilized oocytes release their content into the perivitelline space, the consequence being a proteolytic cleavage of ZP2 protein and inhibition of additional sperm binding to ZP. This modification of ZP is termed zona reaction/zona hardening (Liu, 2011).

A large increase in the level of ooplasmic Ca^{2+} is the key signaling event upon fertilization that, apart from the cortical reaction, triggers also the meiotic cycle resumption, second polar body emission and pronuclear formation. Ca^{2+} is released from the endoplasmic reticulum in regular spikes that persist for about two hours and initiate development. Sperm-specific phospholipase C zeta (PLCζ) that is transferred into the egg at fertilization, induces such Ca^{2+} oscillation and increases IP_3 levels to support this process. A regular pattern of Ca^{2+} oscillation stimulates CaMKII, which is able to inactivate MPF and release the oocyte from MII arrest by stimulating a cyclin B degradation pathway (Berridge et al., 2000; Fan and Sun, 2004; Takahashi et al., 2013). Repeated Ca^{2+} spikes are also needed for an adequate level of APC/C, which then continuously degrade cyclin B until the entry to S phase (Nixon et al., 2002). After maternal and paternal pronuclei fusion the Ca^{2+} oscillation ceases but is resumed again to trigger the mitotic events at the end of the first cell cycle (Berridge et al., 2000).

3.2.2 Preimplantation embryonic development

The activation process of development induced by fertilization occurs sequentially prior to the first cleavage – that is, in most mammals, within the first hour after fertilization (Florman and Fissore, 2014). There is a rapid decrease in MPF activity that correlates with oocyte activation (Fan and Sun, 2004). Along with cortical reaction and block to polyspermy, rapid paternal chromatin decondensation begins. Later on, maternal chromatin decondenses as well and male and female pronuclei are formed (Florman and Fissore, 2014). Formation of pronuclei correlates with a decrease in activity of MAPK (Fan and Sun, 2004). Before embryonic genome activation (EGA), that is before embryo starts to synthesize its own mRNA and proteins, development relies on maternal proteins and mRNAs that are stored in the cytoplasm of the oocyte during oogenesis. These products drive basic biosynthetic processes, direct the first mitotic divisions, and prepare the embryo for activation of its genome and becoming totipotent (Bogliotti and Ross, 2015).

Oocyte-to-embryo transition involves the migration and fusion of the maternal and paternal pronuclei at the 1-cell stage termed zygote (E1.0), genetic and epigenetic reprogramming, first mitotic cleavage and EGA (Ramathal et al., 2015). The transition to cleavage is caused by the activation of MPF (Gilbert, 2000). Mouse embryos take about three and a half days to develop from the 1-cell stage to the blastocyst stage containing 32 or more cells. While the first cell cycle takes 16 – 20 hrs, the second one is completed in the next 18 – 22 hrs. The lengthened cycle from the 2- to the 4-cell stage may be related to EGA (Sakkas and Vassalli, 2017). This particular transition is, in mice, commonly known to occur at 2-cell (E1.5) stage and referred to as a major genome activation, but the process has been shown to be rather gradual than being a single event, with at least four periods of major gene induction, corresponding to the late 1-cell stage, early 2-cell stage, late 2-cell stage, and 8-cell stage (Latham, 2001). However, the first transcription events are detected in the paternal pronucleus as early as prior to pronuclear fusion and referred to as minor genome activation (Duranthon et al., 2008).

At the 8- to 16-cell (E2.0) stage the embryo undergoes compaction and progresses towards utero-tubal junction and further to the uterine environment. This is when the first morphological differentiation occurs due to adhesive interactions between

the blastomeres generation a tightly organized and less distinct mass of cells. The most significant event that occurs following compaction is the differentiation in two distinct cell lines. Cleavage divisions allocate cells to the inside of the developing morula, and these then become the embryoblast (or the inner cell mass – ICM) which is going to form the embryo itself. The outer cells differentiate into the trophectoderm (TE), which subsequently forms the placenta (Marcho et al., 2015). A recent study showed that cells are destined to form either TE or ICM as soon as in the 8-cell stage based on their contractility (Maître et al., 2016).

With further mitotic divisions at the stage of about 32 cells, the embryo develops into a blastocyst and the blastocoel cavity starts to form. This is due to fluid influx into the extracellular spaces mediated by trophectodermal cells (Sakkas and Vassalli, 2017). Preimplantation embryo development through the blastocyst stage is almost completed by the time embryos enter the uterus (E4.0). After the E4.5, embryo hatches from surrounding ZP and subsequently implants in the uterus at the beginning of E5.0 (Yoshinaga, 2013).

3.3 Epigenetics during oogenesis and early embryonic development

As our notion about epigenetics rises, it becomes obvious that these mechanisms play a very important role in reproductive biology. The most profoundly known epigenetic mechanism involved in reproduction is perhaps the inactivation of X chromosome by DNA hypermethylation. Epigenetic changes that include mainly DNA methylation and histone modification, are reversible, but still inherited across generations. These changes are termed germline-dependent. DNA methylation changes lie in specific methylation of selected regions of DNA, namely the cytosine residues proximal to guanines (CpG) serving as a predominant target. The change involves attachment of the methyl group from a donor S-adenosyl methionine to the fifth carbon residue on the cytosine and is therefore referred as 5meC (5-methylcytosine). This modification is associated with transcription repression and gene silencing (McCarthy a Rissman, 2014). *De novo* DNA methylation is carried out by methyltransferases DNMT3A and DNMT3B complexed with DNMT3L, (Kim and Costello, 2017), whereas maintenance of DNA-methylation patterns in proliferating cells is ensured with the activity of DNMT1. Both DNMT1 and DNMT3A

have been shown to interact with histone deacetylases (HDACs) and can repress transcription (Li, 2002).

Histones comprise a nucleosome with DNA wrapped around it and there are 5 of them: core histones H2A, H2B in duplicates, H3 and H4 in duplicates, together forming an octamer, and H1, that serves to link the cores to each other. About 25 % of the histone mass is composed of N-terminal tails. In addition to DNA methylation, core histones undergo post-translational modifications that change the structure of chromatin and are far more complex. They most commonly involve methylation and/or acetylation of lysine residues, but can also occur *via* proline isomerization, sumoylation, ubiquitination, phosphorylation, ADP ribosylation, deamination, and a myriad of combinations thereof. Conformational changes of N-terminal tails *via* protein modifications lead to increased or decreased density of the chromatin structure, therefore different accessibility for transcription factors is achieved and genes are either enhanced or silenced (McCarthy and Rissman, 2014). For example, methylation of lysine 4 of histone H3 (H3K4me) has been associated with active gene expression and is a marker of euchromatin, whereas di- and trimethylation of lysine 9 of histone H3 (H3K9me2/3) has been associated with transcriptional silencing and is a marker of heterochromatin (Li, 2002), as well as the methylated lysine K27 on histone H3 (Bannister and Kouzarides, 2011; Rivera and Ross, 2013). There is a great variety of enzymes that induce and/or maintain these modifications, categorized as histone transferases (McCarthy and Rissman, 2014).

3.3.1 Oocyte epigenetics

An extensive number of epigenetic reprogramming events are taking place during oocyte development, and they, in the first instance, erase the pre-existing epigenetic profile of the somatic cells from which germ cells are specified in the early embryo. After this complex demethylation and erasure of epigenetic marks, the epigenetic patterns of the mature gametes are established once again. Thus, by the time PGCs have migrated to the genital ridge, it is thought that they are largely devoid of DNA methylation (Tomizawa et al., 2012), ten-eleven translocation (TET) family proteins being the largely responsible ones for an active demethylation process (Kim and Costello, 2017).

In the mouse female germline, the period that DNA methylation is re-established is after birth during oocyte growth; *i. e.*, in oocytes arrested in the diplotene stage of prophase I (Tomizawa et al., 2012). These broad-scale DNA methylation events mainly related to CpG are completed by the immature GV stage and remain stable throughout oocyte maturation. However, oocytes accumulate some DNA methylation patterns also during the maturation process. These changes comprise the non-CpG methylation and are established through a generalized process that is not focused on particular genomic regions or functional features (Yu et al., 2017).

Histone modifications (namely the methylation levels of H3K9 and H3K27) in migrating PGCs at E7.0 are similar to those in surrounding somatic cells until the E8.0 when H3K9me2 significantly decreases. Subsequently, the H3K27me3 repressive mark is upregulated by E9.5. The global changes in repressive marks in migrating PGCs might reflect the reprogramming of the PGC genome, which is eventually necessary for the zygote to acquire totipotency (Sasaki and Matsui, 2008). Histone modification levels then rise during the time-course of oocyte growth, where histone acetylation follows a similar time course as DNA methylation. (Albertini, 2014). As the oocyte increase in size, modifications such as H3K4me3 and H3K9me3 gradually increase as well (Rivera and Ross, 2013). The acetylation of mouse histones H3 and H4 have been well documented and in general, all of the examined lysine residues are acetylated in fully-grown oocytes in GV stage (Gu et al., 2010). When the meiotic division is resumed, dramatic loss of histone H3 and histone H4 acetylation was observed prior and during GVBD, suggesting a requirement for histone deacetylation in GVBD and for efficient chromosome segregation. This deacetylation is maintained until the MII stage (Ivanovska and Orr-Weaver, 2006; Gu et al., 2010). In contrast to acetylation and also phosphorylation, histone methylation seems to remain unchanged and relatively stable throughout meiosis (Sasaki and Matsui, 2008; Gu et al., 2010).

The whole concept of epigenetic changes during oocyte development is an important part of the phenomenon termed genomic imprinting. Genomic imprinting is an epigenetic process by which the male and the female germ line confer a sex-specific mark (imprint) on germline differentially methylated regions (gDMR) on the DNA. As a consequence, the paternal and the maternal genome are functionally nonequivalent

and both are required for normal embryonic development. Genomic imprints are erased and newly established during gametogenesis, and stably inherited through somatic cell divisions during post-zygotic development (Horsthemke and Ludwig, 2005). Oocyte has to acquire its crucial epigenetic modifications necessary for development beyond the preimplantation period during the late growth phase (Bao et al., 2000).

3.3.2 Epigenetics of early embryonic stages

Fertilization and the zygote

After the fusion of sperm and egg plasma membranes, sperm nucleus gets into the ooplasm, and ooplasmic factors then modify sperm nuclear structure and protein composition, which is referred to as sperm chromatin remodeling (Nevoral and Sutovsky, 2017). The sperm chromatin undergoes series of morphological changes including its dispersion around the time of oocyte completion of anaphase II, further chromatin recondensation during completion of oocyte telophase II and final decondensation in synchrony with the oocyte chromatin that decondenses in the separate female pronucleus (McLay and Clarke, 2003). Female genome changes its structure immediately after completion of meiosis II and along with the male one become surrounded with pronuclear envelope, a process being associated with rapid decrease of MAPK (Nevoral and Sutovsky, 2017).

These changes are accompanied by the replacement of sperm protamines by histones provided by the oocyte, and the whole process is highly ATP-dependent. Protamines P1 and P2 are sperm-specific basic proteins complexed with DNA, holding a six-fold more compact structure than metaphase chromosomes (McLay and Clarke, 2003). Protamine removal is facilitated by oocyte-produced glutathione that reduces intramolecular disulphide bonds, and further proteasomal activities or nuclear factors (Nevoral and Sutovsky, 2017).

Protamine to histone replacement is followed by active demethylation of the paternal genome and both are completed within first 8 hrs after fertilization before replication begins (Rivera and Ross, 2013; Suter and Aagaard, 2014). There are indirect and direct pathways to achieve active DNA demethylation, that are more and less risky

for the genome at this developmental stage, respectively. An indirect way of demethylation lies in DNA repair mechanism, where 5meC is deaminated to thymine by deaminase, and the mismatched thymine is then excised from DNA strand, resulting in replacement of 5meC with a new cytosine (Morgan et al., 2005; Iqbal et al., 2011). Direct active demethylation is performed through oxidation of 5meC by the maternally stored TET3 enzyme, which is detectable in oocytes, zygotes but not anymore in 2-cell embryos. The newly produced 5-hydroxymethylcytosine (5hmeC) is no longer able to interact with repressor proteins known to bind to 5meC and is not a substrate for the maintenance DNMT1, it is, therefore, diluting the CpG methylation during replication. Levels of 5hmeC in paternal pronucleus are profoundly different from the maternal one. 5meC oxidation products are then gradually lost during preimplantation development due to dilution by cell division, being detectable until the 8-cell stage (Iqbal et al., 2011; Shen et al., 2014; Bogliotti and Ross, 2015)

On the contrary, maternal genome is demethylated over much longer time-course and mostly passively, however it has been shown that TET3 and 5hmeC are present in the maternal pronucleus as well, demonstrating a certain level of active demethylation in the maternal genome (Shen et al., 2014). After the formation of the zygote, passive demethylation relies on replication process during each cell division cycle and is complete by the blastocyst stage. This is hypothesized to be caused by the exclusion of DNMT1 from the nucleus and progressive dilution of methylation marks (Rivera and Ross, 2013)

The general chromatin asymmetry of maternal and paternal pronuclei is maintained by different histone modifications as well. Paternal histone modifications are acquired in a progressive manner after fertilization, ultimately heading to paternal chromatin state that is equivalent to the maternal one, concurrently acquiring its unique marks (Morgan et al., 2005). Some initial differences are apparent – paternal chromatin-associated histones H3 and H4 are much more acetylated than those associated with maternal chromatin, possibly by the availability of these in the cytoplasm (Adenot et al., 1997). On the contrary, methylation levels of the maternal genome are much higher, with H3K4me2/3 and H3K27me2/3 being predominant compared to the paternal genome with only monomethylated lysine residues. H3K9me3 is also detected extensively in the maternal genome, whereas only monomethylated H3K9 occurs in the male pronucleus. The monomethylation pattern is symmetric in both pronuclei

(Lepikhov and Walter, 2004; Santos et al., 2005; Beaujean, 2014). The absence of trimethylated H3, as well as the extensive monomethylation of H4, suggests that paternal chromatin is not divided into hetero- and euchromatin (Van Der Heijden et al., 2005). The delay of paternal genome in acquiring trimethylation of H3K9 is despite a presence of appropriate histone H3K9 methyltransferase (HMT), SUV39H, which is recruited by heterochromatin protein (HP1α) to act in a positive feedback loop and further trimethylate monomethylated lysines (Santos et al., 2005). This methyltransferase is one of the first identified, with specific targeting of H3K9me2 and me3 with bounded HP1 (Bannister and Kouzarides, 2011). HP1β is present in the paternal pronucleus despite missing its target H3K9me2/3 and it is thought that binding of HP1β to monomethylated H3K9 prevent it from conversion to H3K9me3 by SUV39H (Santos et al., 2005). The asymmetry in the H3K9me3 persists up to the four-cell stage (Liu, 2004), whilst levels of H3K27me2/3 and H3K4me2/3 are equalized by the two-cell stage (Beaujean, 2014). The asymmetry of the latter diminishes as the male pronucleus incorporates maternal H3 histones (Lepikhov and Walter, 2004).

Demethylation of both genomes is crucial for accessibility of genes for transcription and to restore totipotency (Morgan et al., 2005). Not all of the methylation marks inherited from the gametes are, however, demethylated, and imprinted genes are protected from this global demethylation wave with modifications of coherent histones – methylated imprinting control regions (ICR) are associated with histone H3 di- and trimethylation on lysine K9, histone H4 trimethylation on lysine K20 and absence of histone H3 acetylation, whereas unmethylated ICR alleles are possibly preserved by methylation of lysine K4 on histone H3 (Feil, 2009). Maternal factor STELLA is also able to protect DMRs from global TET3 mediated demethylation (Nakamura et al., 2012).

After specific genome remodeling, the pronuclei enter S-phase, upon its completion their pronuclear envelopes are disassembled, the chromosomes become condensed and pronuclei subsequently fuse. Embryo cleavage is then initiated with first mitotic division followed by cytokinesis (Nevoral and Sutovsky, 2017).

Embryo cleavage and EGA

With continuous embryo cleavage, DNA methylation is lost progressively and is dependent on DNA replication, resulting in unequal sister chromatids methylation (Morgan et al., 2005). Also, beginning at the 1-cell stage, the EGA is clearly evident by the 2-cell stage, a commonly addressed time of mouse maternal-to-zygotic transition. The timing of this event is highly species-specific and serves three functions. First one is to destroy oocyte-specific transcripts, the second facilitates replacing maternal transcripts that are common to the oocyte and early embryo with zygotic transcripts, and the third is to reprogram the early embryo by generating novel transcripts that are not expressed in the oocyte (Marcho et al., 2015). Most of the maternal mRNA is degraded during the EGA through their adenylation and by microRNA (Nevoral and Sutovsky, 2017).

In mouse, highly accessible, transcriptionally competent chromatin seems to underlie EGA as soon as during the minor wave of EGA in paternal pronucleus. Histone exchange is a general mechanism during embryogenesis, as gamete-specific variants are replaced by somatic versions. This process could mediate gradual nucleosome unpacking prior to EGA, as maternal histones are diluted in favor of permissive zygotic versions. Thus, a globally permissive chromatin conformation is a prerequisite for EGA, which is shaped in part by dynamic incorporation of embryonic histone variants. However, the specificity of activation likely requires local changes to chromatin accessibility (Lee et al., 2014). Changes in TF content and activity are also required. Protein synthesis is essential for genome activation during both early and later phases of transcriptional activation (Latham, 2001).

CpG regions with 5meC maintained by DNMT1 are important in the timing of specific zygotic genes transcription in a way that inhibition of this methyltransferase causes premature expression of many zygotic genes during EGA. Repressive interactions with methyl-CpG-binding domain proteins are necessary to regulate transcriptional timing at EGA. Conversely, DNA hypomethylation is generally predictive of genes expressed at EGA and the deposition of permissive H3K4me3 marks.

Considering histone modifications, there are two types of them associated with specifying gene expression during EGA – lysine acetylation and lysine (tri)methylation. Transcription permissive lysine acetylation on H4 is present in the paternal pronucleus

during minor EGA, but due to HDAC activity becomes transient and undetectable in the 2-cell stage. Thus, replication-dependent deacetylation serves for transcriptional specificity during the major wave of EGA (Lee et al., 2014) Similarly, lysine methylation patterns on H3 influence timing of gene activity during EGA. Whereas H3K27me3 is present in inactive maternal pronucleus, it appears in the transcriptionally competent paternal one only at the end of minor EGA, thus seems to regulate early mouse gene activity (Santos et al., 2005). Variants of histone H2A are also involved in genome reprogramming after fertilization. Phosphorylated foci of H2A (γH2AX) used during preimplantation development are enriched in the early paternal pronucleus. Generally, it marks an early response to DNA damage and double-strand breaks and is therefore important for genome stability. However, it may be independent of DNA damage in the male pronuclei, as tumor suppressor p53-binding protein 1, which is also normally recruited to damage sites, does not colocalize with these foci (Burton and Torres-Padilla, 2014).

Various factors are essential for precise specification of histone marks in the embryo as well as complementing with transcripts that the maternal contribution lacks. The Polycomb Repressive Complex 1 and 2 (PRC1, PRC2) are important in the facilitation of H3K27 methylation and when nonfunctional, embryo stops its development in 2-cell stage during the EGA (Lee et al., 2014). Ubiquitination of H2A is also dependent on PRC1; this modification is relevant with its maintenance and modulation of heterochromatin through facilitation of H3 methylation (Nevoral and Sutovsky, 2017).

Post-translational modifications of histones are either maintained up to the blastocyst stage (H3K9me, H3K27me) or completely disappear from interphase blastomeres. Some of the modifications, such as H3K36me3, reappear, usually after embryonic genome activation, and persist in the blastocyst stage or at implantation. Finally, some specific dynamics also exist – H4K20me is intense up to the 8-cell stage, but then becomes significantly reduced from the morula to blastocyst stage. (Beaujean, 2014).

TE and ICM lineage specification and the blastocyst

The first lineage allocation occurs at the morula stage and leads to the formation of two distinct cell lines of TE and ICM. This is critical for implantation and successful development. Specific transcription factors determine the lineage potential, with OCT4, NANOG and SOX2 being enriched in the ICM to promote pluripotency and resist differentiation (Marcho et al., 2015) Expression of these transcription factors is usually associated with permissive histone modifications enrichment and, conversely, repressive histone variants depletion at their promoter regions (Beaujean, 2014). The possibility that epigenetic asymmetry functions also as a driver for lineage specification has been shown as soon as in the 4-cell stage, where one blastomere of four had lower levels of H3R26me2 and was then more likely to develop into the TE derivatives (Burton and Torres-Padilla, 2014).

Global differences concerning DNA methylation have been found between the extraembryonic (TE) cell mass and the embryonic one (ICM). These differences arise as soon as in the blastocyst stage when the combinations of active and passive demethylation result in a low state of methylation in the TE. On the other hand, the ICM shows clear signs of extensive *de novo* methylation, which may begin as early as in the late morula stage. This is likely due to the presence of *de novo* DNMT3B in the ICM but not in the TE (Morgan et al., 2005).

When the DNA methylation has been shown to be dispensable for growth and differentiation of the extraembryonic lineages, appropriate histone modifications may provide key epigenetic information directing gene expression and lineage specification (Marcho et al., 2015). As with DNA methylation, higher levels of specific repressive histone methylation marks are also found in the ICM when compared with the TE. Methylation of H3K27 is generally more abundant in the ICM compared to TE, where this modification is detected only in the inactivated X chromosome. H3K9me3 marks the heterochromatin foci in ICM (Morgan et al., 2005). On the contrary, phosphorylation of histones H4 and H2A is present in higher levels in the extraembryonic cells (Burton and Torres-Padilla, 2014).

Last but not least, X chromosome inactivation counts as the major epigenetic event of the early embryonic development, involving DNA methylation as well as histone modifications in a synergistic manner. X-chromosome inactivation compensates eventual inequality in gene dosage in males and females. One of the chromosomes, either maternal or paternal, is randomly transcriptionally inactivated and remains so throughout all further cell divisions in the life of an individual. In mice, paternal X-chromosome inactivation is first seen at the blastocyst stage in TE cells, and random inactivation follows in the ICM (Lyon, 1999). X inactivation is under control of upregulated gene *Xist* which transcripts coat the soon-to-be inactivated chromosome. This induces series of epigenetic changes affecting histones and cytosine methylation of the chosen chromosome, histone H3K9 dimethylation and K27 trimethylation being characteristic for the process (Rougeulle et al., 2004). PRC1 and PRC2, as well as G9a histone methyltransferase and CDYL protein, are associated with these modifications, leading to facultative heterochromatin formation (Escamilla-Del-Arenal et al., 2013). These histone modifications then further recruit DNMTs that methylate the DNA strand directly, and X-chromosome inactivation is maintained (Galupa and Heard, 2015).

Epigenetic regulations are a delicate process and when altered, cell death does not necessarily occur. However, the consequences of such epigenetic shift are no less bothersome. Epigenetic-mediated inadequate gene expression can result in idiopathic causes of disease, disturbed gene imprinting, and transgenerational inheritance of altered epigenetic patterns. Endocrine disruptors are indeed the ones that pose a risk for correct epigenetic programming.

3.4 Endocrine disruptors

Endocrine disruptors (EDs) are natural or synthetic chemical substances that mimic or antagonize the action of endogenous hormones and therefore cause hormonal imbalances in animals and humans. ED is defined by WHO as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (Bergman et al., 2013). Many compounds that already exist or are being newly introduced

in the environment have estrogenic, anti-estrogenic or anti-androgenic effect in the organism – that includes groups of chemicals such as insecticides, herbicides and fungicides, plastics and plasticizers, surfactants, industrial chemicals such as polychloro biphenyls (PCBs), polybrominated biphenyls (PBBs) and dioxins, flame retardants, pharmaceuticals, and phytoestrogens (such as genistein and coumestrol) (Zama and Uzumcu, 2010). In 2007, the European Commission introduced a preliminary priority list of substances for further evaluation of their endocrine disrupting effects comprising 428 substances in total, where 194 of them showed a clear evidence of endocrine disrupting effects, 128 showed a potential evidence of ED effects and 109 showed either no scientific basis for inclusion in the list or insufficient data to decide about. Many different groups of chemicals figure on the list, including those with clear evidence of endocrine disrupting effects: alkylphenols and its derivatives, benzoates, chlorinated paraffins, phthalates, dioxins/furans, triazines or PCBs (Commission of the European Communities, 2007).

Endocrine disruptors can act *via* nuclear receptors, non-nuclear steroid hormone receptors (membrane estrogen receptors), non-steroid receptors (neurotransmitter receptors such as the serotonin, dopamine or norepinephrine receptors), orphan receptors, enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that converge upon endocrine and reproductive systems (Diamanti-Kandarakis et al., 2009). Because hormones themselves act in very low concentrations through high-affinity receptors and the response increases in a logarithmic manner until the point of saturation, very low concentrations of environmental EDs could add to the endogenous hormone effect to produce a response that is much greater than would be predicted based on the hormone alone. Effect of ED can be tissue- and cell-specific, since the receptor abundance in different cells is a major determinant of the ability to activate the receptors. In addition to low dose responses, non-linear and non-monotonic responses are also typical for EDs, wherein the latter the slope of the curve changes sign over the course of the dose-response (Bergman et al., 2013).

One of the most harmful effects of EDs lies probably in their ability to interfere with cell and tissues programming during the critical developmental windows when there may be an increased susceptibility to EDs, and thus their effect is expected to be permanent. For reproductive functions in both humans and animals, fetal life is the most vulnerable because there are rapid structural and functional events, and their disruption may not be apparent until the puberty when the functions become activated (Diamanti-Kandarakis et al., 2009).

Moreover, EDs are able to influence not only the exposed individual but also the subsequent generations (F1 – F4) when the germline is affected. This transgenerational inheritance is non-genomic and involves altered epigenetic information (DNA methylation and histone modifications) that is transmitted through the germline in the absence of direct exposure. The critical window for EDs exposure in F0 generation is during the later stages of primordial germ cell migration and colonization of the fetal gonad and coincident with the onset of fetal gonadal sex determination. Effects of EDs interfere with correct DNA demethylation, remethylation and establishment of imprinting during that time, resulting in distinct gDMRs, epimutations, and transgenerational disease inheritance. Such effects had been described using many different compounds including agricultural fungicide vinclozolin, dioxin, the pesticide methoxychlor and a plastic mixture of bisphenol A and phthalates (Anway and Skinner, 2006; Skinner et al., 2013).

3.4.1 Bisphenol A and its effect on mammalian reproduction

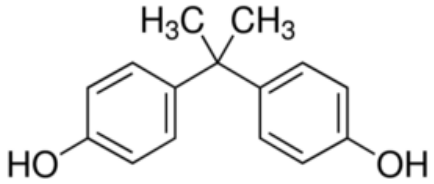

Figure I: ***Chemical structure of BPA****. (source: https://www.sigmaaldrich.com)*

Bisphenol A (BPA) (IUPAC name 4-[2-(4-hydroxyphenyl)propan-2-yl]phenol) is white, crystalline solid chemical substance with a molecular weight of 228.29 g . mol^{-1}. BPA belongs to a group of phenols, which have hydroxyl residue directly bounded to the aromatic ring. The presence of hydroxyl groups in BPA determines its good reactivity.

As other phenols, BPA may be converted to ethers, esters, and salts. Moreover, BPA undergoes electrophilic substitution like nitration, sulphonation or alkylation. It is mainly used in the production of synthetic polymers including epoxy resins and polycarbonates, but because of good mechanical properties, low adsorption of moisture and thermal stability of its polymers, it is widely used in production of variety of items of everyday use such as food containers, bottles, toys, medical equipment, dental products, electronic devices, CD/DVD, thermal paper and recycled paper, polyacrylates, polyesters and lacquer coatings of tins (Michałowicz, 2014). Annual global production of BPA reached up to 5.2 million tons in 2008 and its extensive processing and usage have led to environmental contamination which is strictly connected to anthropogenic activity. Different BPA levels had been detected in the atmosphere, river waters, ground waters, seawaters, sediments, wastewater treatment plant effluents, sewage sludge, soil and indoor dust (Michałowicz, 2014; Björnsdotter et al., 2017). The estimated worldwide release of BPA into the environment is almost half million kg per year (Mileva et al., 2014). Also, BPA monomers get into the ecosystems and foods as a result of diffusion from and hydrolysis of various polymers. Several factors contribute to the enhanced release of BPA, such as decreased pH, increased temperature, mechanical damage or chemical properties of a solution (*e.g.* ethanol admixture) in contact with polymers. Prolonged usage and turnover of plastic containers and bottles is another important contributing factor (Michałowicz, 2014).

The most significant source of human population exposure is the oral route, although BPA can enter the body *via* inhalation and transdermal route as well (Vandenberg et al., 2007; Mileva et al., 2014). Transdermal exposure is a concerning issue when the person is regularly in contact with *e.g.* thermal paper receipts, and the uptake is enhanced if the skin is greasy, freshly washed or the hand cream containing alcohols is applied (Thayer et al., 2016; Björnsdotter et al., 2017)

In the human body, BPA was detected in a multitude of tissues and fluids including blood, serum and plasma, placental tissue, amniotic fluid, follicular fluid, breast milk, saliva, urine, semen (Vandenberg et al., 2007), sweat and adipose tissue (Michałowicz, 2014). BPA is also able to permeate the placenta and therefore poses a risk for fetal development (Jalal et al., 2018).

The major detrimental feature of BPA is its xenoestrogenic activity that is based on chemical structure similarity to 17-β-estradiol (E2). It has the ability to bind to nuclear estrogenic receptors α and β (ERα and ERβ), estrogen-related receptor γ (ERRγ) and also the non-nuclear (membrane) ERs. Although the affinity for nuclear receptors is much lower than the E2 has, the potency to mediate estrogenic responses through membrane ERs is equal to E2. Moreover, it can act as an antiestrogen by competing with endogenous E2. BPA is even able to bind directly to androgen receptors and thus block endogenous androgen action (Rochester, 2013; Jalal et al., 2018). BPA acts as an endocrine disruptor in doses way below the oral reference dose of 50 μg . kg^{-1} . day^{-1}, predisposing an individual to disease especially during critical developmental stages such as prenatal and early life. These effects include *e.g.* increased risk of mammary cancer after prenatal exposure in rodents and altered methylation patterns of several genes expressed in the prostate and mammary glands of rodents when exposed early in life (Seachrist et al., 2016). In addition, aberrant DNA methylation in sperm caused by BPA exposure of gestating females has been shown to be inherited transgenerationally (Desaulniers et al., 2015). Epigenetic mechanisms, in general, are involved in the development of many diseases linked to BPA exposure during clinical and preclinical research (Mileva et al., 2014). Apart from exposure in the critical developmental periods, reference safe daily limit of BPA causes an alteration in the expression of genes also in the adulthood and after short-term exposure. These changes interfered with genes involved in brain prefrontal cortex physiopathology (Castro et al., 2013).

Concerning reproduction, as other EDs, BPA induces detrimental changes in both male and female reproductive functions. For the males, multiple studies in rodents have shown, that perinatal BPA exposure causes permanent changes in the entire HPG axis, has transgenerational effects on testicular steroid hormone receptors and reduces sperm motility and sperm count. The latter effect has been proven also after only six-day exposure in the adulthood. In men, increased urine BPA levels correlate to increased E2, testosterone, and LH levels and decreased sperm motility (Mileva et al., 2014), as well as decreased sperm concentration, count and vitality (Rochester, 2013).

A wide range of evidence is also available for females, with the critical and susceptible windows being those before, during and shortly after the formation

of fertilized eggs, during pregnancy, infancy, childhood, and puberty. BPA can impair the HPG axis on both hypothalamic and pituitary levels by changing the mRNA expression rates of *Kiss1*, GnRH, and gonadotropins. These changes, however, can be manifested both by a decrease and increase depending on BPA doses (Huo et al., 2015). Lately, it has been shown that rats perinatally exposed to low doses of BPA differ with their response to exogenous gonadotropins treatment when in prepubertal age, a finding that coincidence with poor ovarian response to *in vitro* fertilization protocols in women with high BPA concentration in urine and serum (Santamaría et al., 2017).

Hunt et al. (2003) provided one of the first evidence that short-term, low-dose exposure to BPA during the final stages of oocyte growth is able to cause meiotic aneuploidy. Interestingly, studies of Eichenlaub-Ritter et al. (2008) and Pacchierotti et al. (2008) failed to repeat their findings. Conversely, chronic mouse follicle culture exposure to low doses of BPA throughout oocyte growth and maturation was associated with the decreased ability of meiotic resumption after stimulation, increased incidence of cell cycle arrest after GVBD, and also malformed meiotic spindle and chromosome misalignment in both MI and MII oocytes (Lenie et al., 2008). In humans, increasing doses of BPA negatively correlate with the successful maturation of oocytes *in vitro*, with particular infliction of meiotic spindle structure and chromosome alignment (Machtinger et al., 2013).

Effects on porcine oocyte maturation *in vitro* include significantly decreased rates of matured oocytes, abnormal actin distribution, spindle morphology and chromosome alignment; the higher rates of early-stage apoptosis were also detected. Concerning epigenetics, BPA exposure altered methylation levels of histone H3K4 and DNA methylation (Wang et al., 2016). When added to mouse follicle culture, BPA significantly altered methylation of DMRs of maternally imprinted genes in GV oocytes and decreased histone H3K9 trimethylation and interkinetochore distance in MII oocytes (Trapphoff et al., 2013).

In murine embryos, Xiao et al. (2011) have shown the association between relatively high dose BPA treatment (100 mg . kg^{-1} . day^{-1}) and failing embryo implantation, while in lower doses (40 mg . kg^{-1} . day^{-1}) implantation was delayed and perinatal mortality increased. BPA in environmentally relevant doses was, however, not proven as a factor influencing embryo implantation. *In utero* exposure of mouse fetuses to BPA was shown

to induce permanent epigenetic changes that become apparent no before their adulthood. These changes involved altered methylation of genes related to estrogen responses (ERα-binding genes), therefore their different expression was not manifested until sexual maturity when E2 levels rise (Jorgensen et al., 2016).

When all of these evidence of impaired reproductive functions in both animals and humans are taken into consideration (the majority reviewed in Machtinger and Orvieto, 2014; Peretz et al., 2014), it is no surprise that specific actions were taken to protect those that are the most susceptible. European Union banned BPA in baby bottles manufacturing in 2011 (Directive 2011/8/EU) and some EU members (Denmark, Sweden, France, and Belgium) also introduced national bans on using BPA in food contact materials and coatings (Björnsdotter et al., 2017). The U.S. Food and Drug Administration (FDA) banned the use of BPA in baby bottles and children's drinking cups in July 2012 (Liao and Kannan, 2013). BPA has also been banned in thermal paper receipts in Japan (Yamazaki et al., 2015). However, proclaiming plastic products as BPA-free may not necessarily mean that they will not release any other chemicals with xenoestrogenic activity (Bittner et al., 2014). More specifically, bisphenols exist in numerous analogs that can be used instead of BPA for their favorable properties. The most widely used substitution has become the bisphenol S (BPS).

3.4.2 Threat of bisphenol S for human reproductive health

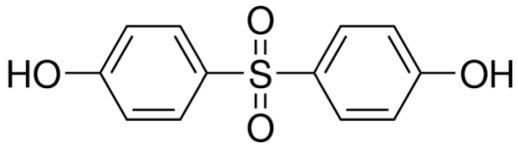

Figure II: ***Chemical structure of BPS****. (source: https://www.sigmaaldrich.com)*

Bisphenol S (BPS) (IUPAC name 4-(4-hydroxyphenyl)sulfonylphenol) is white, crystalline powder with a molecular weight of 250.27 g . mol^{-1}. Along with bisphenol F (BPF), it became the most widespread consumer and commercial replacement for BPA. BPS-based materials confer better heat and organic solvent resistance, increased dimensional stability and better wetting of glass reinforcements. It is an additive

in pesticides, dyestuffs, color-fast agents, leather tanning agents, dye dispersants, and fiber improvers (Molina-Molina et al., 2013). Further industrial applications include BPS as a wash fastening agent in cleaning products, an electroplating solvent, a constituent of phenolic resin and a developer in thermal paper. BPS can be generally expected in all goods where BPA was initially used and it applies so on products proudly tagged as "BPA-free". So far, BPS has been detected in personal care products (body wash, hair care, make-up, lotions, toothpaste), paper (receipts, banknotes, tickets, flyers) and food (dairy and meat, vegetables, canned food, and cereals) (summarized in Rochester and Bolden, 2015). When different types of paper were analyzed, thermal paper receipts were shown as the major source of human exposure to BPS (Liao et al., 2012), that can significantly increase BPS levels in urine and serum in cashiers (Thayer et al., 2016). Among the foods, it is not surprising that the highest overall concentrations of bisphenols were found in preserved, ready-made foods, where the migration from polycarbonate bottles and tin coatings due to structural similarities is expected. However, BPA and BPF were the predominant bisphenols in this category, with the highest concentrations in canned food (Liao and Kannan, 2013).

According to European Chemicals Agency, annual manufactured or imported rate of BPS was as high as 1,000 to 10,000 tons in the European Economic Area. In the environment, BPS already seems to be ubiquitous worldwide, although it does not reach the concentrations of BPA yet. BPS has been detected in river and surface waters, sewage sludge, sediments and indoor dust (Wu et al., 2018), and it appears to be more resistant to environmental degradation when compared to BPA (Liao et al., 2012). Human exposure was demonstrated with detection of this chemical in urine (Wu et al., 2018) and serum (Wan et al., 2018) samples from the U.S. and Asia, with the main pathways to the human body being inhalation, dietary and dust ingestion, and dermal contact (summarized in Žalmanová et al., 2016). The predominant metabolic pathway of BPS in humans is glucuronidation (Skledar et al., 2016).

BPS has been proven to have xenoestrogenic potencies in the same order of magnitude as BPA. *In vitro,* BPS interacts with both human nuclear ERs (ERα and ERβ) and also with androgen receptor (Molina-Molina et al., 2013). These interactions generally lead to decreased levels of androgen and increased levels of estrogen and progestogen

(Rosenmai et al., 2014). Moreover, a non-genomic interaction of BPS in low doses (femtomolar to picomolar) with membrane-bound ERs was also shown in rat pituitary cell line, interfering with E2 signaling and resulting in altered cell proliferation, apoptosis, and prolactin release (Viñas and Watson, 2013). BPS and some of its metabolites exhibited agonistic estrogenic activities also in yeast assay system (Skledar et al., 2016).

Numerous studies were performed using zebrafish (*Danio rerio*) as an experimental model to demonstrate adverse effects of BPS. Developmental exposure have led to increased expression of reproductive-related genes and receptor system KISS/KISSR (an upstream regulator of GnRH neurons) (Qiu et al., 2016), as well as to activation of expression of specific estrogenic marker (aromatase B gene) in larvae brain (Cano-Nicolau et al., 2016). Experiments of Naderi et al. (2014) and Ji et al. (2013) on developing and adult zebrafish, respectively, both concluded imbalances in steroid hormones with an increase of E2 in both males and females, decreased testosterone in the males and delayed hatching with lower rates. F1 generation was also affected. Moreover, Naderi et al. (2014) showed shifted sex ration in favor of females, decreased egg production and sperm count and imbalance of thyroid hormones. More recently, the estrogenic potential of BPS was again demonstrated on different tissues and cells throughout developmental stages (Le Fol et al., 2017).

Kinch et al. (2015) showed that low-dose BPS exposure in zebrafish resulted in 240% increase in neurogenesis within the hypothalamus, a similar increase as with exposure to BPA. Environmentally relevant BPS levels also modulated immunity-related gene (Qiu et al., 2018) and microRNA expression (Lee et al., 2018), and exhibited obesogenic effects by interfering with lipid metabolism on multiple levels (Wang et al., 2018).

Experiments performed on *Caenorhabditis elegans* showed alterations in gene expression of, among others, genes associated with embryo development ending in birth or egg hatching, reproduction, nematode larval development, and growth. Also, BPS caused reproductive defects such as germline apoptosis and embryonic lethality (Chen et al., 2016).

In mammals, a limited amount of studies has been performed so far. In mice, BPS at doses of 10, 50 and 100 µg . kg bw^{-1} . day^{-1} was shown to significantly decrease

fertilization rates after *in vitro* fertilization, and blastocyst rate reduction was significant with the same doses. Development of embryos was significantly inhibited only at doses of 50 and 100 μg . kg bw^{-1} . day^{-1}. Moreover, BPS exposure was also associated with oxidative stress in ovarian tissue (Nourian et al., 2017). The idea of pregnancy and lactation being sensitive to xenoestrogen exposure was supported with a study showing a reduced fraction of milk-producing units of the mammary gland in late lactation and also altered nursing behaviors of mice and pups after BPS exposure (LaPlante et al., 2017). Conversely, evaluation of BPS effects on bovine granulosa and *theca* cells brought significant results as an increase in basal E2 secretion by bovine granulosa cells only at 100 μM BPS levels. This high concentration is however very unlikely to occur naturally (Campen et al., 2018).

Finally, a study on so far the closest animal model to human reproduction had provided rather disturbing results. Complex set of low-dose BPS effects was evaluated on porcine oocytes – firstly, BPS treated oocytes significantly postponed or failed to achieve MI and MII stages under *in vitro* conditions. Meiotic spindle organization of such oocytes necessary for correct chromosome segregation was severely disturbed with 3 nM BPS treatment. Further, used BPS concentrations (3 nM, 300 nM and 30 μM) had an impact on quantities of ER and aromatase transcripts, their protein distribution and amounts, and altered the hyaluronic acid production during cumulus cell expansion (Žalmanová et al., 2017).

The whole group of effects of BPA and its structural analog BPS on mammalian organism raises the question whether BPS can be a safe substitution to BPA. Available materials concerning BPS action are scarce and those taking epigenetics into account are still missing. EDs induced changes in the epigenome can be acquired silently over a long time-course and with subsequent transgenerational effect. The impact on the next generations due to epigenetic mechanisms is therefore likely even with slightly altered epigenetic factors after the exposure.

Based on all of the mentioned evidence, it is hypothesized that BPS has an effect on female reproductive functions with specific regard to oocyte and embryo quality and epigenetics, and results are demonstrated on the murine model.

4 Materials and Methods

4.1 Chemicals and BPS dosing

All chemicals were purchased from Sigma-Aldrich (USA) if not stated otherwise. The exposure scheme consisted of four BPS (BPS1 – BPS4) concentrations and vehicle control (VC). The stock solution (0, 0.004, 0.375, 37.5 and 375 μg . ml^{-1}) was prepared by dissolving BPS in ethanol which was then added to drinking water into final concentrations of BPS 0, 0.004, 0.375, 37.5 and 375 ng . ml^{-1}; the ethanol concentration did not exceed 0.1% (v/v) and vehicle control consisting of pure ethanol was prepared, in accordance with Del Moral et al. (2016). The exposure of mice was carried out using five BPS doses: 0, 0.001, 0.1, 10 and 100 ng . g bw^{-1} . day^{-1} *i. e.* vehicle control (VC) and BPS1 – BPS4 groups, respectively. BPS was administered through drinking water. Selected doses included the EFSA and NIH limits for intake of BPA, a structural analogue of BPS which is now substituted with BPS in many applications.

4.2 Animals, dosing and hormonal stimulation

All animal procedures were conducted in accordance with Act No 246/1992 Coll., on the Protection of Animals against Cruelty, under the supervision of the Animal Welfare Advisory Committee at the Ministry of Education, Youth and Sports of the Czech Republic, approval ID MSMT-11925/2016-3. Four-week-old CD-1 (ICR) mice were purchased from Velaz Ltd. (Czech Republic), housed in intact polysulfonate cages and maintained in a facility with a 12L:12D photoperiod, a temperature 21±1 °C and a relative humidity of 60 %. A phytoestrogen-free diet 1814P (Altromin) and ultrapure water (changed twice per week) were provided ad libitum.

Immediately after admission to the Animal Research and Care Facility, mice were randomly assigned to the experimental groups and allowed to acclimate for one week. BPS was then administered to the mice for 4 weeks as described above. The chosen window of exposure corresponds to at least three waves of cyclic follicle recruitment, beginning at the time of initial recruitment and remaining until the attainment of the reproductive peak (McGee and Hsueh, 2000; Moore-Ambriz et al., 2015).

After four-week exposure, 5 IU of eCG was administered intraperitoneally, 5 IU hCG was administered 46 – 48 hrs later. Concurrently with hCG administration, mice destined for embryo flushing were mated.

Alternatively, mice were euthanized by cervical dislocation 16 hrs later and ovulated cumulus-oocyte complexes were yielded from oviduct. Complexes were treated with 0.1% hyaluronidase in M2 medium and used for further experiments.

4.3 *In vivo* fertilization assay and embryo flushing

Following eCG/hCG treatment of females treated as described above, the *in vivo* fertilization assay was performed using aforementioned natural mating. Embryos were flushed from the oviduct and fertilization rate was calculated as the percentage of cleaved two-cell embryos of total recovered embryos/oocytes. Two-cell embryos were flushed from oviduct at E1.5, blastocyst enclosed in *zona pellucida* were flushed from the uterus at E4.5. The experimental design is shown in Figure III.

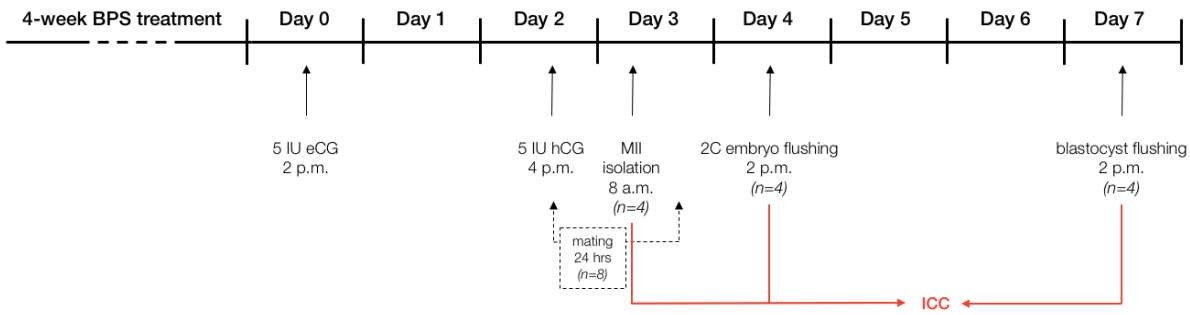

Figure III: ***Experimental design scheme.*** *Hormonal stimulation and fertilization following BPS treatment are indicated along with the timing of single samples isolation. Subsequent methodological approach (immunocytochemistry) is shown with red arrows. The number of mice per group used in a particular experiment is indicated as* n.

4.4 Fixation of oocytes/embryos and preparation for ICC

Oocytes intended for staining of α-tubulin and pericentrin were fixed in 4% PFA with 0.1% PVA in PBS for 30 mins. Oocytes and embryos subjected to staining of H3K4me2, H3K9me3, ubH2A, γH2AX, and HP1 were permeabilized with 0.1% PBS-PVA

with 0.03% Tween20 for 20 secs. Fixation was then performed using 4% PFA-PVA-TX-Tween (4% PFA in PBS + 0.1% PVA + 0.04% Triton X-100 + 0.3% Tween20) for 15 mins in 37 °C. Samples fixed by both of these protocols were then washed in 0.1% BSA in PBS with 0.01% Tween 20 and permeabilized using 0.1% Triton X-100 in PBS for 15 mins.

Alternatively, embryos stained for 5meC and H3K27me2 were fixed in 4% PFA with 0.1% PVA in PBS for 30 mins, washed in 0.1% BSA in PBS with 0.01% Tween 20 and permeabilized using 0.1% Triton X-100 in PBS for 15 mins. After further washing, embryos were incubated with 2M HCl with 0.1% PVA for 30 mins, neutralized in Tris-HCl (100mM Tris/HCl buffer with 0.1% PVA) for 10 mins, washed in 0.1% PBS-PVA with 0.03% Tween20 and incubated in 0.25% (w/v) trypsin for 1 min. at 37°C. Samples were then washed in 0.1% BSA in PBS with 0.01% Tween20.

4.5 Immunocytochemistry (ICC)

After blocking of all samples in 0.1% BSA in PBS with 0.01% Tween20 for 15 mins, the 1 hr incubation with specific antibodies followed: anti-α-tubulin (Sigma, USA), pericentrin (PCNT; Abcam, UK), anti H3K27me2 (H3K27me2; Abcam, UK), anti-5'-methyl cytosine (5meC; Abcam, UK), anti H3K9me3 (H3K9me3; Abcam, UK), anti H3K4me2 (H3K4me2; Abcam, UK), anti ubH2A (ubH2A; Abcam, UK), anti γH2AX (H2AX; Abcam, UK) and anti HP1 (HP1; Abcam, UK). Thereafter, samples were washed and incubated for 1 hr with the cocktail of anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 647. Concurrently with final washing, oocytes stained for α-tubulin and pericentrin were treated with phalloidin for β-actin visualization. All samples were mounted on microscope slides in Vectashield with DAPI (Vector Laboratories, H-1200) and stored until imaging at 4 °C.

4.6 Microscopy and image analysis

Confocal microscope with the spinning disc (Olympus, Germany) was used for imaging in VisiView® software (Visitron Systems, Germany). Images were then analyzed in ImageJ software (NIH, Bethesda, USA) and the signal intensity (*i. e.* integrated density) of markers in view was measured. Integrated density is expressed as the product of

Mean Gray Value (an average value of all selected pixels) and their area. Signal intensity of experimental groups was expressed as the integrated density of single markers and related to control groups.

4.7 Statistical analysis

The data were processed with the Microsoft Excel. For overall comparison of the study groups, single factor ANOVA was used. In case of a significant overall finding, differences between individual group pairs were assessed post-hoc using t-test. The level of statistical significance was set at $\alpha = 0.05$ and all reported P-values and tests were calculated as two-tailed.

5 Results

5.1 Influence of BPS on meiotic spindle structure and chromosome alignment in MII oocytes

During this experiment, the aim was to evaluate an effect of BPS on structural properties in matured oocyte that play a role in correct chromosome segregation and meiosis completion (α-tubulin in the meiotic spindle, pericentrin in MTOCs), as well as on chromosome alignment in the metaphase plane. Four different concentrations of BPS were tested in this experiment (0.001, 0.1, 10 and 100 ng . g bw^{-1} . day^{-1} as BPS1 – BPS4 groups, respectively). Immunofluorescence staining was used for this purpose with subsequent imaging on confocal microscope.

Increased number of abnormal oocytes with severely disturbed spindle architecture was observed in BPS treated groups. The microtubules were not correctly, or at all, attached to the chromosomes and the shape of the spindle was malformed. The chromosomes were misplaced and not correctly aligned. Significant number of such abnormal oocytes was present in every BPS-treated group except from BPS1 when compared to controls. Pictures are shown in the Figure IV.

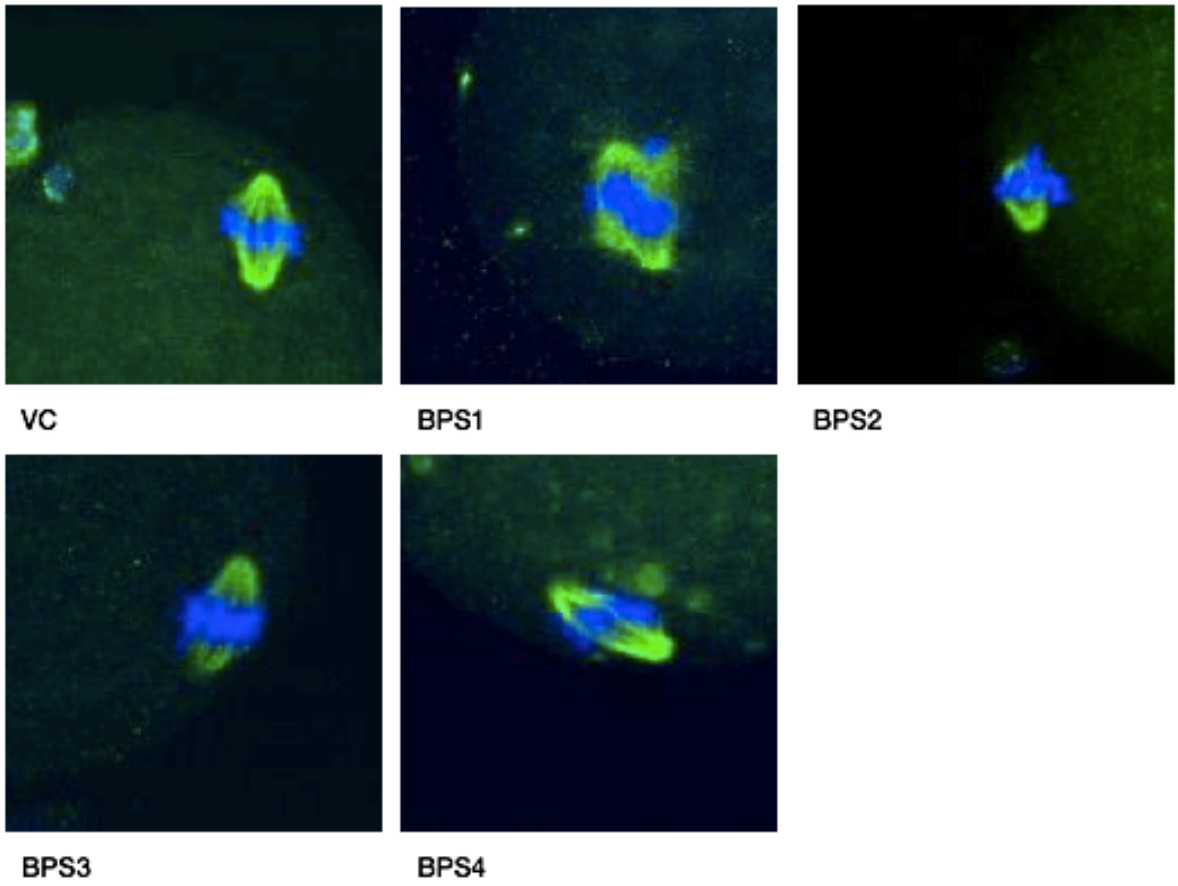

Figure IV: ***Meiotic spindle and chromosome visualization in MII oocytes.*** *α-tubulin of the spindle is shown in green, chromosomes are blue-coloured. Each of BPS-treated group is indicated as BPS1-4 (0.001, 0.1, 10 and 100 ng . g bw^{-1} . day^{-1} BPS concentrations, respectively) including control oocytes (VC).*

These findings of oocyte malformations were further supported with pericentrin (PCNT) staining, where BPS treatment resulted in externally placed PCNT in BPS1 group, cap-shaped PCNT poles in BPS2 group and divided PCNT poles in BPS4 group. These changes are indicated in Figure V.

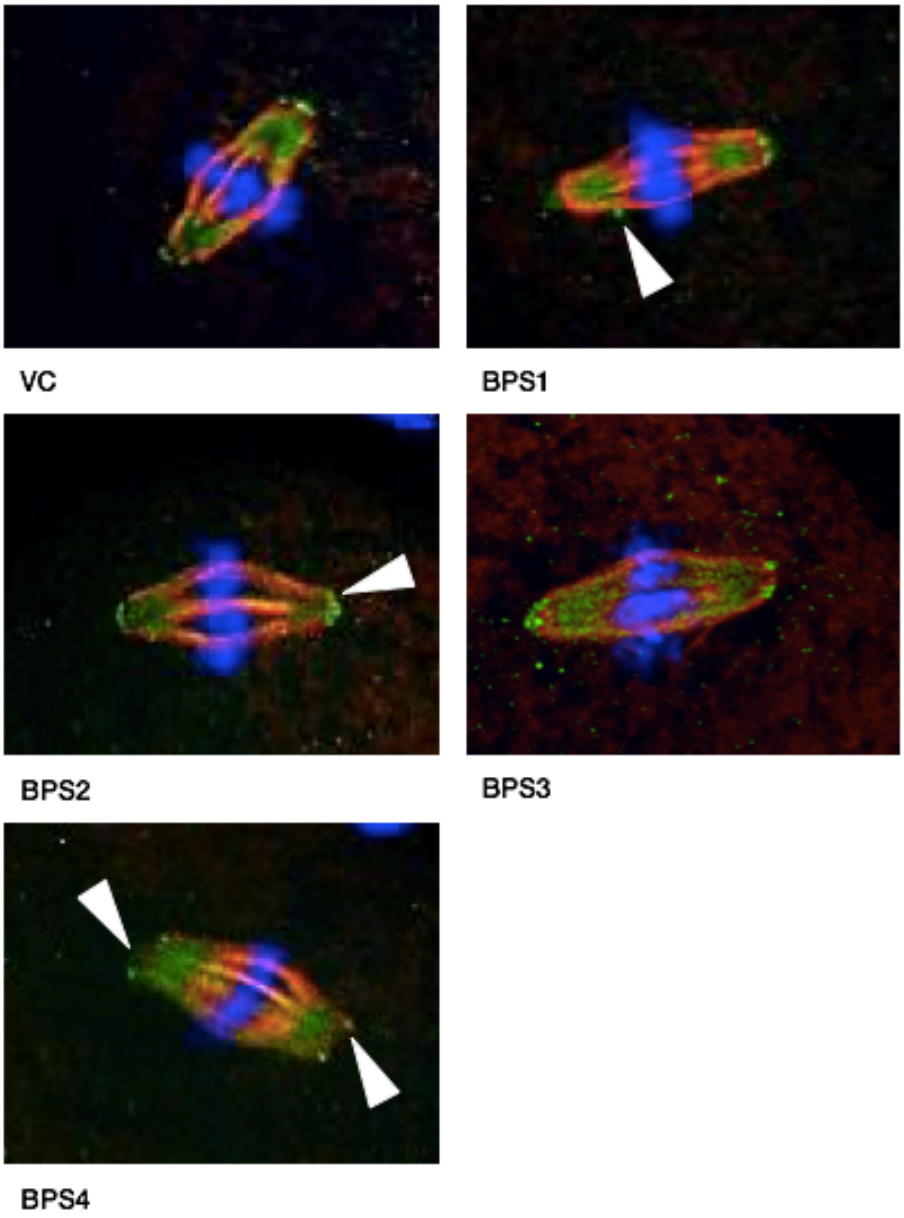

Figure V: ***Meiotic spindle, MTOCs and chromosome visualization in MII oocytes.*** *α-tubulin of the spindle is shown in red, pericentrin signal stains green, chromosomes are blue-coloured. Abnormalities in pericentrin signal are indicated with white arrowheads. Single BPS-treated groups are inscribed as BPS1-4 (0.001, 0.1, 10 and 100 ng . g bw^{-1} . day^{-1} BPS concentrations, respectively) including control oocytes (VC).*

Since the most profound and severe changes were observed in BPS2 group, this particular BPS concentration has been used alone as the very low, most interesting and potentially damage-causing one in the majority of further experiments.

5.2 Influence of BPS on genome-wide methylation in MII oocytes

The effect of BPS on epigenetics was evaluated in MII oocytes using specific staining for 5meC. This repressive modification should remain relatively stable during maturation since it establishes specific methylation patterns to the oocyte. Four different BPS concentrations were used (0.001, 0.1, 10 and 100 ng . g bw^{-1} . day^{-1} as BPS1 – BPS4 groups, respectively) and the effect was compared to the vehicle control (VC) group. As in the previous experiment, immunofluorescence staining was used followed by confocal microscopy imaging and signal intensity analysis in ImageJ software.

The statistically significant difference ($p < 0.05$) was only found between BPS2 group and the BPS3 and BPS4 groups. There was no significant effect ($p > 0.05$) in any BPS treated group compared to control group. Results are shown in Figure VI.

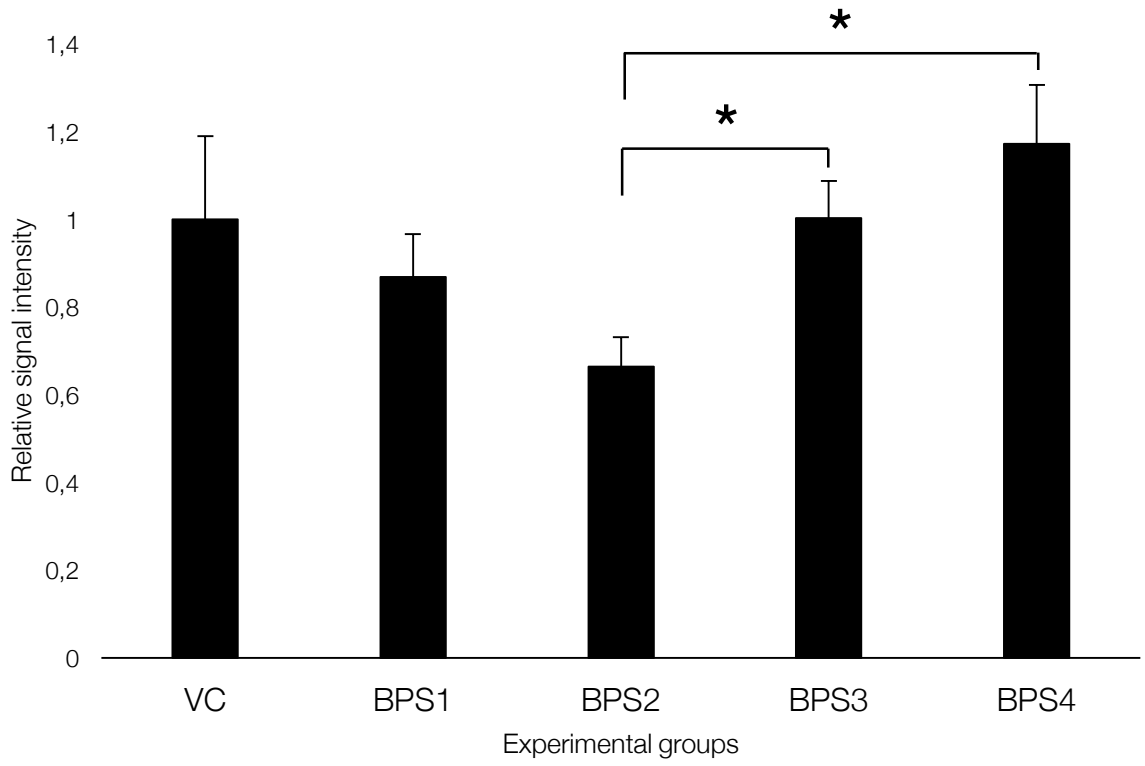

Figure VI: ***Quantification of genome-wide methylation in MII oocytes.*** *Y-axis shows average relative fluorescence signal intensity of 5meC ± SEM. X-axis marks different experimental groups. VC = control group, BPS1-4 = 0.001, 0.1, 10 and 100 ng . g bw^{-1} . day^{-1} BPS concentrations, respectively, * = statistically significant difference ($p < 0.05$).*

5.3 Influence of BPS on H3K4me2, H3K9me3, and H3K27me2 in MII oocytes

The aim of this experiment was to evaluate an effect of 0.1 ng . g bw^{-1} . day^{-1} BPS concentration (BPS2) on methylation levels of histone H3 lysine residues K4, K9 and K27 in murine metaphase II oocytes, whose levels should remain constant throughout oocyte maturation.

Statistical analysis showed no significant differences between H3K9 and H3K27 methylation of control (VC) and BPS treated (BPS2) oocytes ($p > 0.05$). However, there were statistically significant differences in H3K4 dimethylation levels between BPS2 and control oocytes ($p < 0.05$). Results are shown in Figure VII.

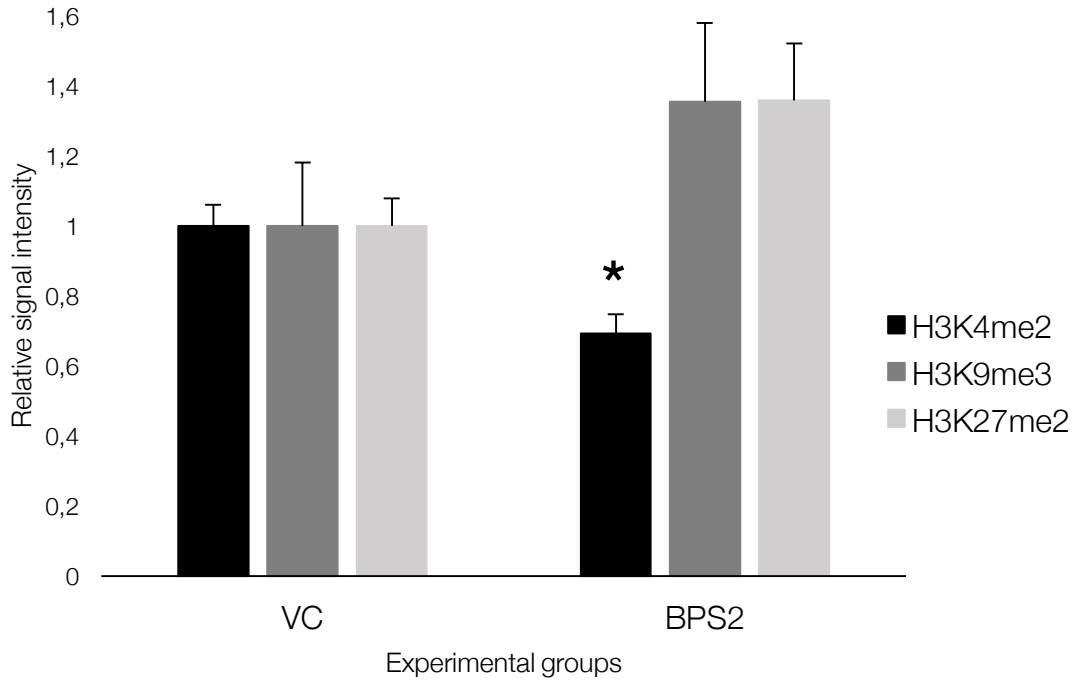

Figure VII: ***Quantification of H3K4, H3K9 and H3K27 methylation levels in MII oocytes.*** *Y-axis shows average relative fluorescence signal intensity of a given marker in oocytes ± SEM. X-axis marks different experimental groups. VC = control group, BPS2 = 0.1 ng . g bw^{-1} . day^{-1} BPS concentration, * = statistically significant difference ($p < 0.05$).*

5.4 Influence of BPS on genome-wide methylation in 2-cell embryos

This experiment was designed to quantify an effect BPS poses for DNA methylation in murine 2-cell embryos, that is important for correct expression of embryonic genes at this developmental stage. Four different BPS concentrations were used (0.001, 0.1, 10 and 100 ng . g bw^{-1} . day^{-1} as BPS1 – BPS4 groups, respectively) and the effect was compared to control (VC) group.

The statistically significant difference ($p < 0.05$) was found between the controls and embryos treated with BPS2 concentration (0.1 ng . g bw^{-1} . day^{-1}). Effect of the other concentrations of BPS was not significant compared to control group. Significant differences were also found between groups BPS1 and BPS2, BPS2 and BPS4, and BPS3 and BPS4 ($p < 0.05$). Results are shown in Figure VIII.

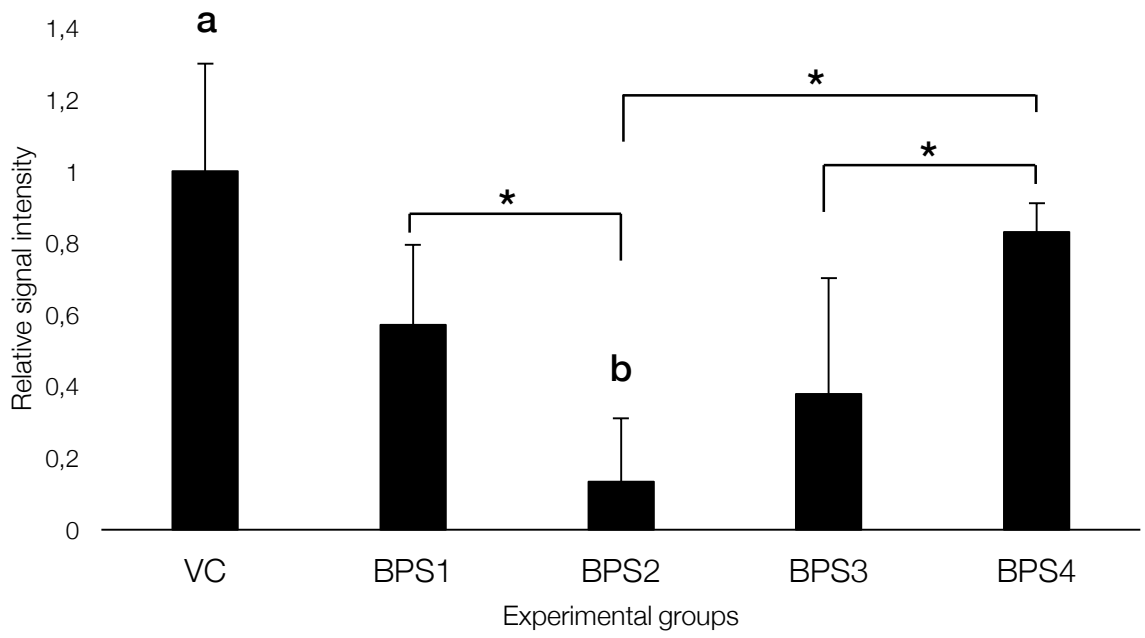

Figure VIII: ***Quantification of genome-wide methylation in murine 2-cell embryos.*** *Y-axis shows average relative fluorescence signal intensity of 5meC ± SEM. X-axis marks different experimental groups. VC = control group, BPS1-4 = 0.001, 0.1, 10 and 100 ng . g bw^{-1} . day^{-1}BPS concentrations, respectively, a/ b/ * = statistically significant difference ($p < 0.05$).*

5.5 Influence of BPS on H3K4me2, H3K9me3, and H3K27me2 in 2-cell embryos

The aim of the experiment was to quantify an effect of very low BPS concentration on di-/trimethylation levels of histone H3 lysine residues K4, K9 and K27 in mouse 2-cell embryos. Histone H3 methylation levels play an important role in hetero- and euchromatin establishment and correct progression through EGA.

A statistically significant effect was observed in H3K9me3 after BPS2 (0.1 ng . g bw^{-1} . day^{-1}) treatment of embryos compared to controls ($p < 0.05$). Concerning H3K4me2 and H3K27me2, no statistically significant difference was found between BPS treated groups and control groups ($p > 0.05$). Results are shown in Figure IX.

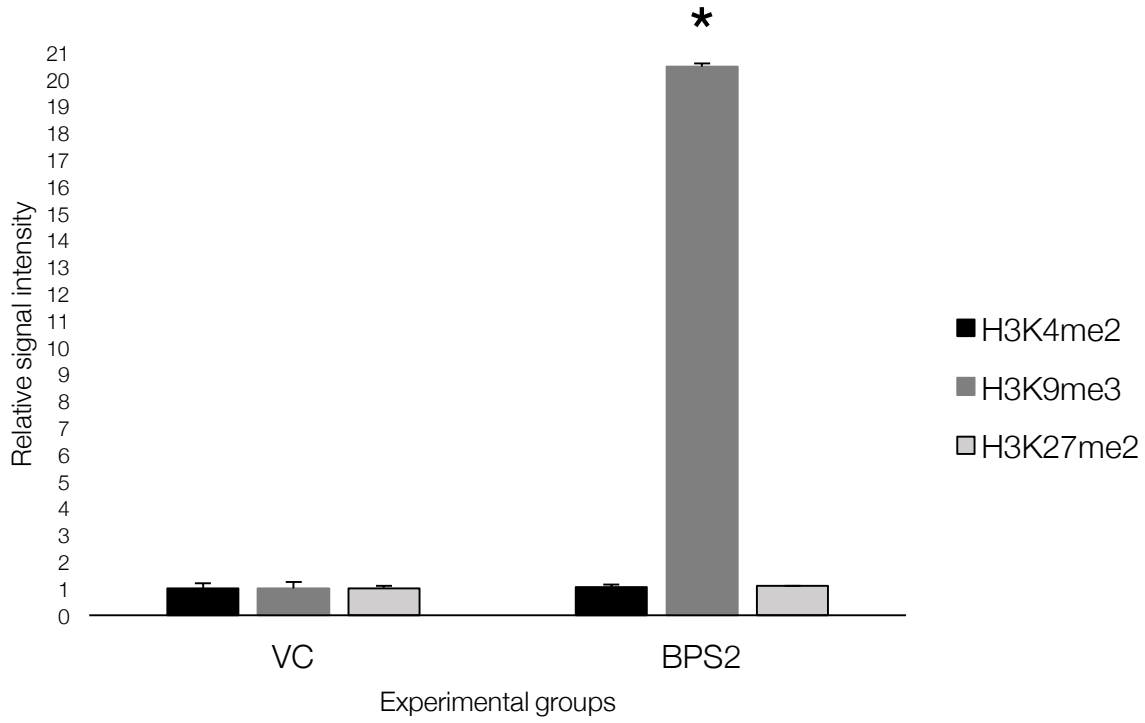

Figure IX: ***Quantification of H3K4, H3K9 and H3K27 methylation in murine 2-cell embryos.*** *Y-axis shows average relative fluorescence signal intensity of histone methylation ± SEM. X-axis marks different experimental groups. VC = control group, BPS2 = 0.1 ng . g bw^{-1} . day^{-1} BPS concentration, * = statistically significant difference ($p < 0.05$).*

5.6 Influence of BPS on ubH2A in 2-cell embryos

In this experiment, BPS2 concentration (0.1 ng . g bw^{-1} . day^{-1}) was used to evaluate an effect of this ED on histone H2A ubiquitination using immunofluorescence staining, confocal microscopy imaging and signal intensity analysis. Ubiquitination levels of H2A are relevant in histone H3 methylation and heterochromatin forming. No significant effect of BPS was found in the level of ubH2A in 2-cell embryos ($p > 0.05$). Results are shown as Figure X in Enclosures.

5.7 Influence of BPS on blastocyst formation and quality

During this experiment, BPS2 concentration (0.1 ng . g bw^{-1} . day^{-1}) was used to evaluate the effect of this chemical in murine blastocysts. The BPS2 dosage was selected as an actual exposure dosage of mankind with significant impact earlier been found in the aforementioned experiments. Based on the previous observations, four different epigenetic markers (H3K4me2, H3K9me3, HP1, and γH2AX) were tested in single blastomeres, along with the number of blastomeres per blastocyst to see its developmental potential. Heterochromatin protein 1 (HP1) is an important marker of heterochromatin, whereas phosphorylated histone H2AX foci (γH2AX) are an early marker of DNA damage and their increased number positively correlate with apoptotic changes in the cells.

Surprisingly, BPS affected ($p < 0.05$) the number of blastomeres per blastocysts, strikingly with a higher number of blastomeres in BPS2 treated group. Results showing blastomeres counts are available in Figure XI.

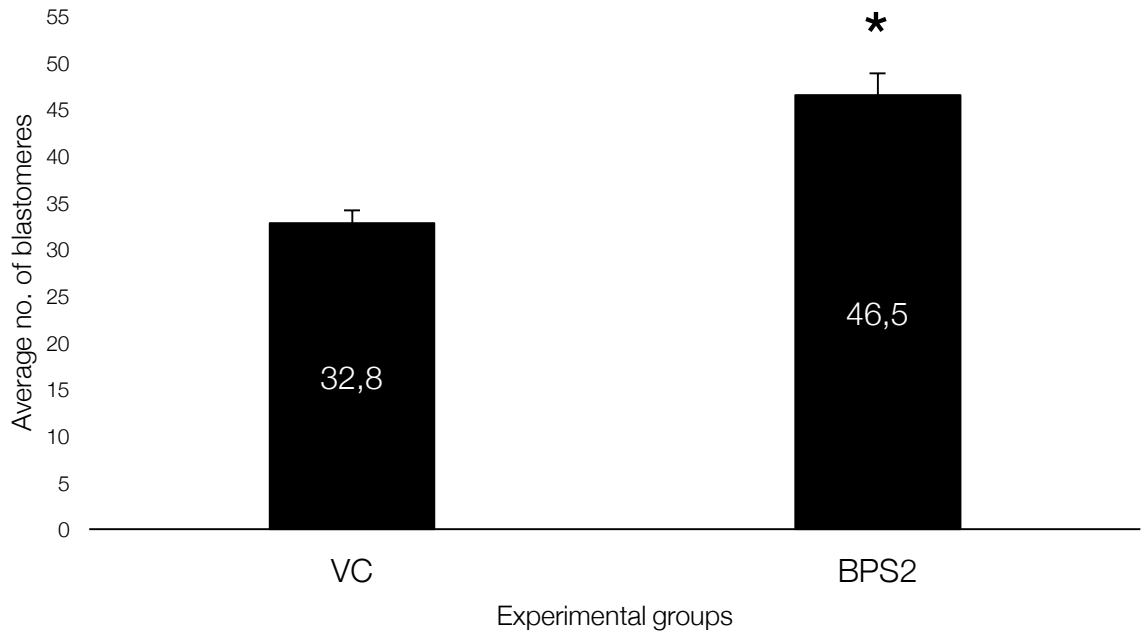

Figure XI: ***Numbers of blastomeres per blastocyst.*** *Y-axis shows the average number of blastomeres ± SEM, X-axis represents experimental groups. VC = control group, BPS2 = 0.1 ng . g bw^{-1} . day^{-1} BPS concentration, * = statistically significant difference (p < 0.05).*

After evaluation of apoptotic changes in single blastomeres, it was obvious that there are almost no differences between control and experimental group concerning an average number of apoptotic blastomeres per blastocyst, and the average γH2AX foci count in apoptotic blastomeres. Although the percentages of apoptotic blastomeres per average total counts of blastomeres were distinct, this difference was not statistically significant (p > 0.05). Results are shown in Table I.

Table I: ***Average number and percentage (%) of blastomeres per blastocyst (blm/blc), apoptotic (phosphorylated H2AX foci positive) blastomeres per blastocyst (AP blm/blc), and average number of H2AX foci per apoptotic blastomere (H2AX foci/AP blm) ± SEM.*** *VC = control group, BPS2 = 0.1 ng . g bw^{-1} . day^{-1} BPS concentration, avg = average, n = number, AP = apoptotic, blm = blastomere, blc = blastocyst, SEM = standard error of the mean.*

	VC	% out of avg n blm	BPS2	% out of avg n blm
blm/blc	32.8 ± 1.326		46.5 ± 2.343	
AP blm/blc	13.89 ± 2.348	41.14 ± 7.624	13.12 ± 1.946	28.78 ± 3.878
H2AX foci/AP blm	1.81 ± 0.192		1.71 ± 0.170	

Although the levels of H3K4me2 were distinct between control and BPS2 treated blastocysts, the effect was not statistically significant. Concerning H3K9me3, detection of this particular histone modification failed, perhaps due to poor binding of antibodies.

Profound changes have been found in HP1 amounts between control and BPS treated blastocysts, with much higher signal intensity in BPS2 group ($p < 0.05$). Results are shown in Figure XII.

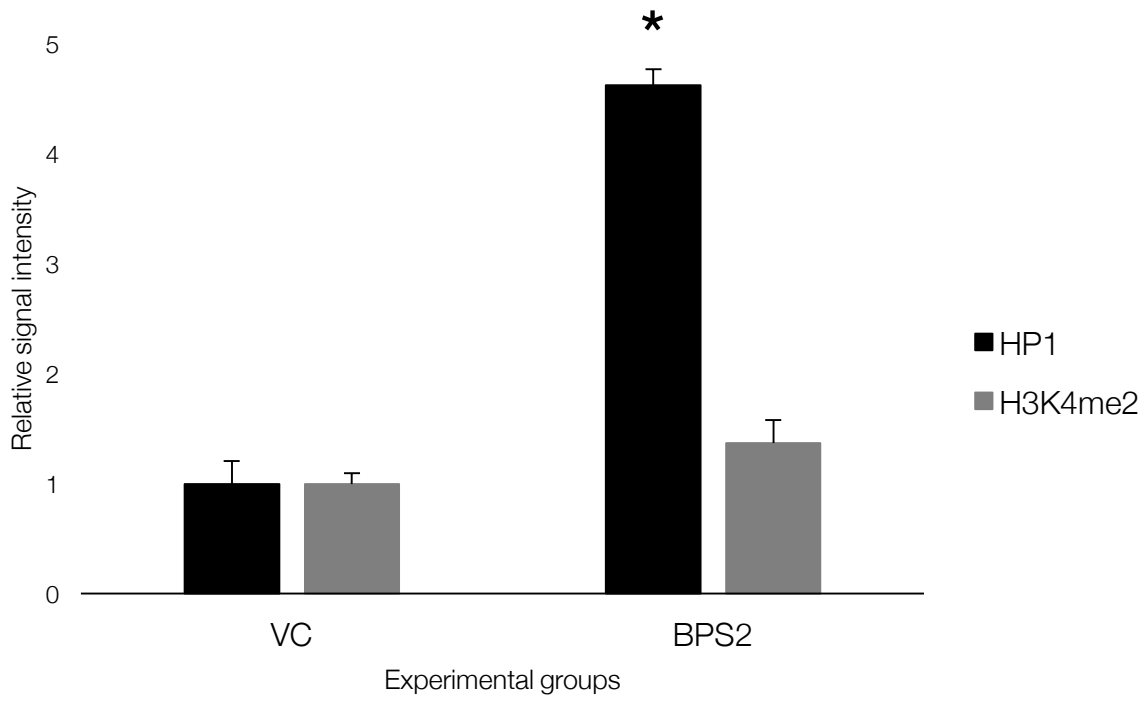

Figure XII: ***Quantification of H3K4 dimethylation levels and HP1 abundance in murine blastocysts.*** *Y-axis shows average relative fluorescence signal intensity of H3K4me2 and HP1 in single blastomeres ± SEM. X-axis marks different experimental groups. VC = control group, BPS2 = 0.1 ng . g bw^{-1} . day^{-1} BPS concentration, * = statistically significant difference ($p < 0.05$).*

6 Discussion

The aim of this thesis was to evaluate the effect of bisphenol S (BPS) on mammalian reproduction with special attention to epigenetic markers and meiotic spindle structure. The results are demonstrated on the murine model.

BPS is a potential endocrine disruptor due to its structural similarities to bisphenol A that has been already proven to have an endocrine disrupting effect (Rochester, 2013; Mileva et al., 2014; Wang et al., 2016). BPS commonly replaces harmful BPA in a broad variety of products, it is, therefore, appropriate to evaluate its effects in the mammalian organism. The concentrations used in this thesis experiments are lower than, but also include the tolerable daily intake (TDI) or the oral reference dose (RfD) for BPA established by European Food Safety Authority (EFSA) and Environmental Protection Agency (EPA), respectively, as 50 ng . g bw^{-1} . day^{-1}. Although EFSA has lowered this number to 5 ng . g bw^{-1} . day^{-1} in 2014, it is still one order of magnitude higher than the most harmful concentration observed in submitted results (0.1 ng . g bw^{-1} . day^{-1}). The No Observed Adverse Effect Level (NOAEL) set up by the FDA are even much higher, reaching 5 µg . g bw^{-1} . day^{-1} (Lorber et al., 2015). All these benchmarks, however, apply for BPA, because reference levels for BPS have not been established yet. Used BPS concentrations in this work also resemble the environmentally relevant doses for humans (Wu et al., 2018).

The exposure window was designed to cover the time of initial recruitment and at least three waves of cyclic recruitment of the follicles, which corresponds to the puberty onset and regular hormonal cycles of the reproductive peak in humans (Moore-Ambriz et al., 2015). This period is considered to be very sensitive to endocrine disrupting chemicals action because of its dependence on correct hormonal signaling (Huo et al., 2015).

Treatment with four different concentrations of BPS has shown a negative impact of this chemical on meiotic spindle architecture and chromosome alignment in oocytes arrested in metaphase II. Spindle formation is crucial for oocyte meiotic maturation and fertilization. These results are in accordance with previous studies of BPA effects in porcine and murine oocytes both *in vitro* and *in vivo* (Hunt et al., 2003; Lenie et al.,

2008; Wang et al., 2016). Although some of the experiments with BPA failed to reproduce these effects on meiotic spindle and chromosome alignment in statistically significant numbers, they proved the influence on misplaced pericentrin foci in accordance with the results in this thesis (Eichenlaub-Ritter et al., 2008). Lately, the similar effect has been observed also directly for low doses of BPS in porcine oocytes matured *in vitro* (Žalmanová et al., 2017).

The mechanism by which BPS is able to interfere with spindle organization is not known, but there are multiple pathways it can affect. In general, BPS can be expected to somehow influence the meiotic spindle assembly checkpoints that delay the anaphase progression when the spindle is disrupted and its connection to the chromosomes is compromised (Gorbsky, 2015). The MAPK is present in the spindle and is particularly colocalized with MTOCs, it is therefore expected to be involved in spindle organization during meiosis (Fan and Sun, 2004). The decrease of phosphorylated MAPK has been in association with BPA treatment, and it is suggested that spindle aberrations can be triggered through this pathway (Wang et al., 2016). Moreover, 17-β-estradiol (E2) has been shown to have a detrimental effect on the meiotic spindle when added to culture during *in vitro* maturation (Beker-van Woudenberg et al., 2004), so it might be that E2-additive potential of BPS can lead to impaired spindles as well.

Such disturbances in meiotic spindle structure can lead to aneuploidies, embryonic mortality, and further developmental disorders. In addition, increased number of isolated abnormal oocytes lowers the chance of successful IVF cycles at ART clinics – the negative impact of BPA on this structure has been demonstrated in human *in vitro* matured oocytes as well (Machtinger et al., 2013).

Epigenetic mode of action is a well-known feature of EDs. Consisting of DNA methylation and histone modifications, epigenetic patterns are extensively reprogrammed during the late phases of oocyte growth and the early phases of embryonic development, beginning at fertilization. This allows the oocyte to acquire its specific imprinting marks when modifications are established, and conversely, the broad demethylation and coherent histone modifications during the first cell cycles of embryonic development ensure its correct progression through EGA and acquirement of totipotency. To my best knowledge, there is not any study specifically evaluating effects of BPS on such

epigenetic markers in oocytes and embryos yet, comparison with studies of BPA is therefore still the only option.

After oocyte and 2-cell embryo isolation following the BPS treatment, the genome-wide methylation levels were evaluated. Interestingly, the same pattern of 5meC alterations was observed in both oocytes and embryos, where the second lowest BPS concentration (BPS2) reached the most profound change of decreased methylation. Furthermore, the difference between the control group was statistically significant in 2-cell embryos. These results are in accordance with Dolinoy et al. (2007), who showed that BPA exposure leads to hypomethylation of CpG sites. More recently it has been discovered that altered DNA methylation after BPA treatment comprises the imprinted gene regions (Susiarjo et al., 2013) that are of maternal origin (Trapphoff et al., 2013). Perhaps the most recent proof of decreased DNA methylation induced by BPA was brought up by Wang et al. (2016). While alterations of the 5meC amount in the oocytes can be a marker of insufficient imprint establishment, changes in embryonic genome-wide methylation can ultimately lead to aberrant imprints in the regions associated with developmental imprinting disorders (in case of the ICM) or the abnormal placenta development (in case of TE). This thesis emphasizes the role of extremely low doses of BPS in the induction of methylation changes concerning the concentrations used were 10^2- to 10^5-fold lower than in the studies of Susiarjo et al. (2013) and Dolinoy et al. (2007), respectively.

Methylation of histones further contributes to heterochromatin and euchromatin establishment, depending on its position. This ultimately leads to differential gene expression. In this thesis, dimethylation levels of histone H3 lysine residue K4 (H3K4me2), which supports gene activation, were found to be significantly decreased in MII oocytes compared to controls. This observation is in accordance with a study on porcine oocytes (Wang et al., 2016). Although H3K9me3 and H3K27me2 were conversely increased, their levels did not reach to statistical significance. But, while the H3K4me2 occupies the same promoters of developmentally important genes as repressive H3K27me2 (Bernstein et al., 2006), and this is believed to balance the expression of genes necessary for development (Voigt et al., 2013), found methylation pattern may be unfavorable for the further

developmental process. Further experiments with possibly more replicates could elicit this presumption in the future.

On the contrary, in 2-cell embryos, significant alterations were found only for H3K9me3, while the H3K4me2 and H3K27me2 remained almost unchanged. The H3K9me3 shift lies in the very prominent increase of H3K9me3 in BPS2 group. This histone modification is a repressive one, and it also has the ability to recruit DNMTs for *de novo* methylation and to maintain expression-inactive conformation (Trapphoff et al., 2013). Therefore, such change would be very inappropriate during the ongoing EGA, which relies on permissive chromatin conformation for correct embryonic gene expression and histone variants incorporation (Lee et al., 2014).

Another histone modification in view in 2-cell embryos was the monoubiquitination of histone H2A (ubH2A). This modification participates in histone H3 methylation facilitation and is controlled by the same protein complex as the H3K27me – the PRC (Nevoral and Sutovsky, 2017). Since neither ubH2A nor H3K27me2 were significantly altered in BPS2 group, it can be concluded that this low BPS dose has no effect on their amounts and possibly also on their upstream regulation factors PRC1 and PRC2.

In blastocysts, apart from non-significant differences in H3K4me2 detection and failed detection of H3K9me3, increased amounts of HP1 were detected. This protein binds to monomethylated H3K9 and initiates its further methylation to H3K9me3 (Van Der Heijden et al., 2005; Trapphoff et al., 2013). Such increase (compared to controls) in the HP1 at this developmental stage could be caused by incomplete degradation of maternally stored HP1 during the process of degradation of maternal factors that is necessary for correct EGA progression. On the other hand, the embryonic HP1 is expressed already in the 2-cell stage, so the cause may lie solely in BPS-induced altered gene expression. The observation of increased HP1 also coincides with high H3K9me3 detected in 2-cell embryos. As a consequence, development of such individual can be disturbed due to differently expressed genes because of altered chromatin state. However, it was not possible to tell if the rate of affection is the same, different or restricted only in the TE and/or ICM because of the blastocoel cavity, as well as two cell lineages, were not fully apparent. It can be concluded that BPS2 treatment significantly affects HP1 amount in the whole blastocyst.

Lastly, apoptotic changes marked by phosphorylation of histone H2AX (γH2AX) were analyzed in each blastomere. This histone modification is an early response to DNA double strain breaks, after its occurrence the phosphorylation results in γH2AX foci which extend up to several Mb in mammals. This leads to structural alterations at the damaged site to foster DNA repair (Bonisch and Hake, 2012). Moreover, the γH2AX formation is as well induced by the apoptotic DNA fragmentation and might play a role in the facilitation of packaging the fragmented DNA into apoptotic bodies of somatic cells. Thus, DNA repair and apoptosis probably share common initial chromatin-related steps (Rogakou et al., 2000). The apoptotic rate of blastocyst may significantly increase when DNA damage repair is inadequate. Increased number of foci would, therefore, mark low-quality blastocysts that consist of damaged blastomeres with decreased developmental potential.

There was no statistically significant effect observed in BPS2 treated blastocysts, yet the variance in the percentage of apoptotic blastomeres per blastocyst was almost 13 % between control and BPS treated group, surprisingly in favor for blastocysts after BPS treatment. As the control group showed, the presence of γH2AX foci is not a rare observation and such occurrence can simply mean the DNA strands are held together for efficient repair (Bonisch and Hake, 2012). In the case of decreased foci occurrence in BPS treated embryos, it can also represent an adaptable reaction of the cells to the exposure and recruitment of powerful repair mechanisms stimulated by these low doses. Nevertheless, we can presume that BPS exposure does not trigger changes in apoptotic or DNA repair mechanism. However, this is in contrast with a study on bovine *in vitro* model, where BPA was shown to induce significant apoptotic changes in treated blastocysts (Ferris et al., 2016). The cause of these opposite conclusions may lie in different detection technique used.

Surprisingly, BPS increased the number of blastomeres per blastocyst, an effect that can suggest an improvement in the development of exposed embryos. Yet, consequences of such results might not be as positive as they seem. The fastest-dividing embryos *in vitro* are currently the most attractive for embryo transfer, but this rapid development can also be a marker of attenuated mitotic entry coordinating Chk1-dependent checkpoint. This checkpoint is activated by DNA damage and delays entry into mitosis until DNA repair process is ensured. Chk1-dependent checkpoint can be

weakened *e.g.* by *in vitro* culture conditions that are believed to improve fertilization efficiency (Ladstätter and Tachibana-Konwalski, 2016). Other supporting results were obtained with *in vitro* bovine embryos, where the slow-dividing blastocysts gene expression and metabolic profile were more similar to those produced *in vivo*, a feature that could ensure higher pregnancy rates (Milazzotto et al., 2016). This result should be further confirmed with repeated experiments and possibly more markers involved.

Using mouse experimental model can have several limitations when relating the results to humans. There are multiple differences concerning reproductive functions between mice and humans including dissimilar signaling cascades and oocyte lipid contents, follicular development, sensitivity to exogenous compounds or timing of developmental milestones such as EGA that would suggest using a more similar animal model such as the pig. On the other hand, studies on mice provide unique opportunity to perform experiments *in vivo* (that would be inevitable to confirm *in vitro* results anyway) and imitate the chemical exposure throughout the life of a mammalian individual. Mice are easily available and this can further increase the statistical power of the experiments. There is also an advantage in the rapid generation time and relative ease of mice breeding – it allows performing transgenerational studies that are very important concerning epigenetic mechanisms of EDs (Bohacek and Mansuy, 2017). Such study should be a logical follow-up to the submitted results.

This thesis has shown that BPS exposure prior to the reproductive period of mice (ovulation, fertilization, and development of embryos) can have consequences for quality of the oocytes as well as for the developing embryos. The effects comprise changes in the cytoskeleton of the oocytes and alterations in epigenetics of both oocytes and embryos. Such changes can lead to meiotic aneuploidies, disturbed genomic imprinting and ultimately developmental disorders. In real-life, however, an individual is exposed to a mixture of endocrine disrupting chemicals rather than a single specific one. Evaluation of such combinations is critical to a fundamental understanding of endocrine disruptions and resulting diseases (Viñas et al., 2012; Viñas and Watson, 2013b).

7 Conclusion

This thesis brings novel results concerning adverse potential of bisphenol S to influence the quality of oocytes and embryos and demonstrates them on the murine model. Specific targets of BPS were evaluated, consisting of meiotic spindle structure, chromosomal alignment and epigenetic markers of oocyte and embryo quality.

Two hypotheses were tested – that BPS can affect the meiotic spindle and chromosome alignment, and that BPS influence the epigenetics in oocytes and early embryos. The first hypothesis was confirmed with the very low, environmentally relevant doses affecting the spindle architecture and incidental chromosome misalignment. The most profound aberrations were evident with the concentration as low as 0.1 ng . g bw^{-1} . day^{-1}, which is way below the established tolerable daily intake for humans.

The second hypothesis was confirmed when some of the assessed epigenetic markers of hetero- and euchromatin in both oocytes and embryos were affected. Significant differences were found in genome-wide methylation, histone H3K4 dimethylation, histone H3K9 trimethylation and coherent heterochromatin protein 1 (HP1) amount. BPS also affected the number of blastomeres per blastocyst. The results were manifested in non-linear dose-response manner that confirms the endocrine disrupting effect of such low BPS doses.

Taken together, these findings support a possible role of low doses of BPS in meiotic aneuploidies and transgenerational inheritance due to altered epigenetics that have a strong impact on female reproductive abilities and development of an organism. These effects are very similar to those of BPA, it is, therefore, adequate to consider BPS to be the regrettable substitution for BPA. A rapid action should be taken to estimate the safe reference levels for BPS and assess the risk for human public health, as well as consider negative impacts of such analogues when replacing those already proved as harmful.

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9 List of abbreviations

12L:12D	Photo regimen of 12-hr day
5hmeC	5-hydroxymethylcytosine
5meC	5-methylcytosine
ADP	Adenosine diphosphate
AMH	Anti-Müllerian hormone
APC/C	Anaphase-promoting complex/cyclosome
AR	Acrosome reaction
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BLIMP1	B-lymphocyte-induced maturation protein 1
BMP-2; -4; -7; -15	Bone morphogenetic protein-2; -4; -7; -15
BPA	Bisphenol A
BPF	Bisphenol F
BPS	Bisphenol S
BSA	Bovine serum albumin
CaMKII	Ca^{2+}/Calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
CD9	Cluster of differentiation 9 protein
CDC2	Cell division cycle protein 2
CDC25B	Cell division cycle protein 25B
CDK1	Cyclin-dependent kinase 1
CDYL	Chromodomain Y like
cGMP	Cyclic guanosine monophosphate
CpG	Cytosine proximal to guanine
CSF	Cytostatic factor
DAPI	4',6-diamidino-2-phenylindole
DNMT1	DNA (cytosine-5)-methyltransferase 1
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
DNMT3B	DNA (cytosine-5)-methyltransferase 3B

DNMT3L	DNA (cytosine-5)-methyltransferase 3L
E2	17-β-estradiol
E(7.5)	Embryonic day (7.5)
eCG	Equine chorionic gonadotropin
ED	Endocrine disruptor
EFSA	European Food Safety Authority
EGA	Embryonic genome activation
EGF	Epidermal growth factor
EPA	Environmental Protection Agency
ER	Estrogen receptor
ERK1	Extracellular signal-regulated kinase 1
ERK2	Extracellular signal-regulated kinase 2
ERRγ	Estrogen-related receptor γ
ERα/β	Estrogen receptor α/β
EU	European Union
FDA	Food and Drug Administration
FIGα	Factor in the germline α
FSH	Follicle-stimulating hormone
G9a	G9a histone methyltransferase
GCC	Germ cell clusters
GDF9	Growth differentiation factor 9
gDMR	Germline differentially methylated regions
GFP	Green fluorescent protein
GnRH	Gonadotropin-releasing hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
H1	Histone H1
H2A	Histone H2A
H2B	Histone H2B
H3	Histone H3
H3K27	Histone H3 Lysine 27

H3K27me2/3	Histone H3 Lysine 27 di/trimethylation
H3K36me3	Histone H3 Lysine 36 trimethylation
H3K4	Histone H3 Lysine 4
H3K4me2	Histone H3 Lysine 4 dimethylation
H3K9	Histone H3 Lysine 9
H3K9me2/3	Histone H3 Lysine 9 di/trimethylation
H3R26me2	Histone H3 Arginine 26 dimethylation
H4	Histone H4
H4K20me1	Histone H4 Lysine 20 monomethylation
hCG	Human chorionic gonadotropin
HDAC	Histone deacetylase
HMT	Histone methyltransferase
HP1/ß	Heterochromatin protein 1/β
HPG	Hypothalamic–pituitary–gonadal
ICM	Inner cell mass
ICR	Imprinting control regions
IGF-1	Insulin-like growth factor 1
IP_3	Inositol triphosphate
IU	International unit
IUPAC	International Union of Pure and Applied Chemistry
IVF	*In vitro* fertilization
KISS/KISSR	Kisspeptin/kisspeptin receptor
Kiss1	Kisspeptin 1 gene
KIT	Proto-oncogene tyrosine-protein kinase Kit
KITL	Proto-oncogene tyrosine-protein kinase Kit ligand
KO	Knock out
LH	Luteinizing hormone
Lhx8	LIM Homeobox 8
MAPK	Mitogen-activated protein kinase
MAPKAPK	Mitogen-activated protein kinase-activated protein kinases

MEK	Mitogen-activated protein kinase kinase 1
MI	Metaphase I
MII	Metaphase II
MOS	MOS proto-oncogene protein
MPF	M-phase/maturation promoting factor
mRNA	Messenger RNA
MTOC	Microtubule-organizing center
MYT1	Myelin transcription factor 1
NGF	Nerve growth factor
NIH	National Institute of Health
NOAEL	No observed adverse effect level
NOBOX	Newborn ovary homeobox-encoding
NSN	Non-surrounded nucleolus
OCT4	Octamer-binding transcription factor 4
p53	Tumor protein p53
PBB	Polybrominated biphenyls
PBS	Phosphate-buffered saline
PCB	Polychloro biphenyls
PCNT	Pericentrin
PDE	Phosphodiesterase
PDE3	Phosphodiesterase 3
PDE3A	Phosphodiesterase 3A
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PGC	Primordial germ cells
PH-20	PH-20 hyaluronidase
PKA	Protein kinase A
PLCζ	Phospholipase C ζ
PLX1	*Xenopus* Polo-like kinase
PRC1; 2	Polycomb repressive complex 1; 2
PRDM1	PR domain zinc finger protein 1

PRDM14	PR domain zinc finger protein 14
PVA	Polyvinyl alcohol
RfD	Oral reference dose
RSK	Ribosomal S6 kinase
SD	Standard deviation
SEM	Standard error of the mean
Ser	Serine
SN	Surrounded nucleolus
Sohlh1; 2	Spermatogenesis and oogenesis specific basic helix-loop-helix 1; 2
SOX2	SRY (sex determining region Y)-Box 2
STELLA	Developmental pluripotency-associated protein 3
SUV39H	Suppressor of variegation 3-9 homolog 1
TDI	Tolerable daily intake
TE	Trophectoderm
TET	Ten-eleven translocation gene family
TET3	Tet methylcytosine dioxygenase 3
TF	Transcription factor
TGFß	Transforming growth factor β
Thr	Threonine
Tween20	Polysorbate 20
VC	Vehicle control
WEE1	WEE1 G2 checkpoint kinase
WHO	World Health Organization
WT	Wild type
Xist	X-inactive specific transcript
ZP1; 2; 3	Zona pellucida sperm-binding protein 1; 2; 3
γH2AX	Histone H2AX phosphorylation

10 Enclosures

I.

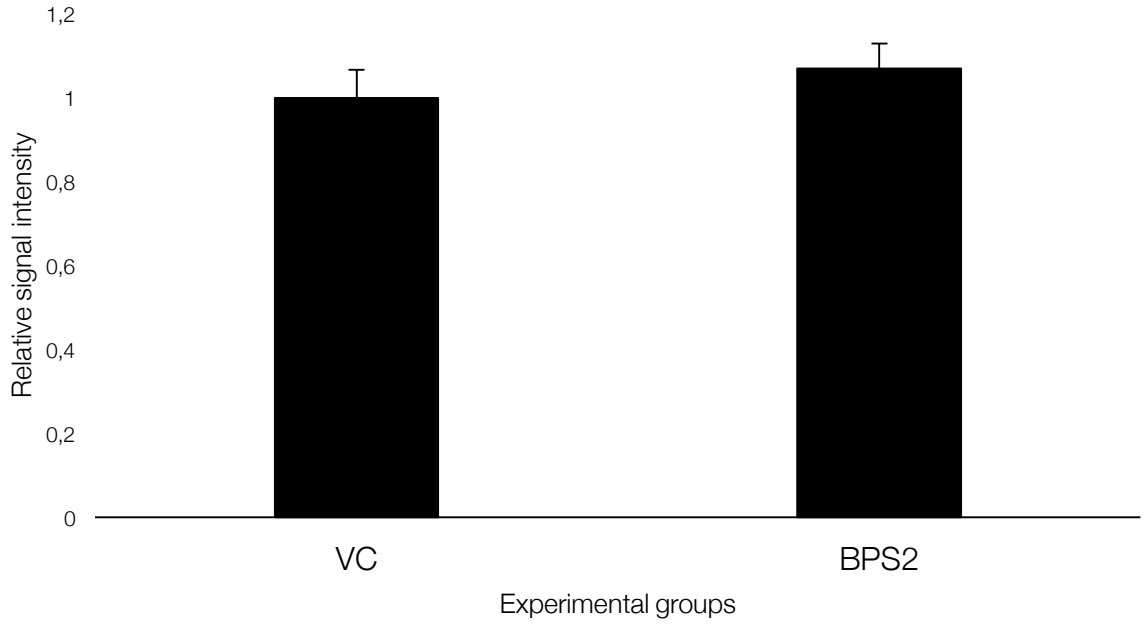

Figure X: ***Quantification of histone H2A ubiquitination in murine 2-cell embryos.*** *Y-axis shows average relative fluorescence signal intensity of ubiquitinated histone +- SEM. - axis marks different experimental groups. VC = control group, BPS2 = 0.1 ng . g bw^{-1} . day^{-1}.*

11 List of enclosures

I. Figure X: Quantification of histone H2A ubiquitination in murine 2-cell embryos