Charles University

Faculty of medicine in Pilsen

Autoreferát dizertační práce

Epigenetic modifications of the sperm and the application in clinical practice of human assisted reproduction therapy

Epigenetické změny spermií a jejich využití pro klinickou praxi v asistované reprodukci člověka

Miriama Štiavnická

Pilsen *2019*

Disertační práce byla vypracována v rámci prezenčního doktorského studijního programu **Ústavu Histologie a Embryologie LF UK v Plzni**.

Uchazeč: **Miriama Štiavnická**

Předseda oborové rady: **prof. MUDr. Milena Králíčková, Ph.D.**

Školitel: **doc. Ing. Jan Nevoral, Ph.D.**

Konzultant: **DVM. Olga García-Álvarez, Ph.D.**

Oponenti: **assoc. prof. DVM. Pei-Shiue Jason Tsai, Ph.D**
National Taiwan University

Ing. Jaromír Vašíček, Ph.D.
Slovak University of Agriculture in Nitra

Mgr. Soňa Kloudová, Ph.D.
Reprofit, Brno

Obhajoba disertační práce před komisí pro obhajobu disertačních prací studijního programu
Anatomie, histologie a embryologie
se koná dne: v: hod.

Místo obhajoby:

Tato disertační práce vznikla za podpory grantu

S disertační prací je možno se seznámit na děkanátě Lékařské fakulty Univerzity Karlovy v Plzni, Husova 3, Plzeň.

ABSTRAKT

Základ zdravého embryonálního vývoje je položen už v průběhu gametogeneze. V současné době, v souladu s nárůstem procenta párů, které nemohu počít dítě přirozenou cestou, centra asistované reprodukce zaznamenávají svůj rozmach. Právě proto, porozumění biologie gamet a toho, co předávají embryu, je klíčem k léčbě neplodných párů a cestou k zdravému embryonálnímu vývoji. Proto se tato studie zaměřuje na epigenetický kód gamet, s prediktivním potenciálem, a na faktory, které epigenetický kód utváří. V souladu s předpokladem, že jsou vybrané molekulární faktory použitelné jako markéry kvality, byl studován vliv environmentálního polutantu bisfenolu S (BPS) na kvalitu zárodečných buněk a spermií s cílem identifikovat markery kvality gamet, použitelné pro screening spermií a jejich selekci pro účely asistované reprodukce. Pro dosáhnutí daných cílů byly použity vzorky lidských spermií, zárodečné buňky experimentálních myší kmene ICR a kančí spermie. Vzorky byly podrobeny analýzám pomocí průtokové cytometrie, immunocytochemie a western blotu. Experimentální práce byla schválena Etickou komisí FN Plzeň, resp. probíhala v souladu se schváleným projektem pokusu na experimentálních zvířatech.

Studie provedená na lidských spermiích detekovala metylovaný histon H3 na lysinu K4 (H3K4me2) jako potencionální epigenetický marker, který je nejen indikátorem kvality spermií, ale i nezralosti chromatinu způsobeného neúplnou protaminací. V druhé části studie jsme prokázali úlohu sulfanu (H_2S) jako antikapacitačního agens, který zpomaluje kapacitaci, pravděpodobně prostřednictvím posttranslačních modifikací proteinů. Dále jsme prokázali úlohu histon deacetylázy SIRT1 napříč maturací oocytu, a identifikovali jeho cílenou relokalizaci ze zárodečného váčku nezralého oocytu do bezprostřední blízkosti dělícího vřeténka maturovaného oocytu. Poslední část studie se zabývala vlivem endokrinního disruptoru BPS na kvalitu gamet, u oocytu byly prokázané malformace dělícího vřeténka a aberace histonového kódu sledovaného pomocí H3K27me2. Co se týče vlivu na samčí reprodukci, BPS efekt se projevil změnou spektra acetylovaných proteinu varletní tkáně.

Předložená práce poskytuje nové epigenetické markery, které lze nadále využít k hodnocení kvality a selekci gamet, určené pro účely asistované reprodukce. Poznatky se tak mohou podílet na zlepšení úspěšnosti mimotělního oplození a zvyšování kvality embryí.

SUMMARY

Basement of healthy embryo development comes from quality of oocytes and spermatozoa. Today, when percentage of couples suffering infertility together with assisted reproductive therapy (ART) is increasing, understanding to gamete biology and heritable epigenetic code is crucial. The study is focused on promising epigenome based markers that could serve as indicators of gamete quality for either their screening or selection for ART. Accordingly selected markers were used for the investigation of environmental pollutant bisphenol S (BPS) effect on gametes quality. To obtain these aims, we have used human semen samples, boar semen samples and ICR mice gametes. Samples were analyzed by flow cytometry, immunocytochemistry and western blot analysis. All experimental work was in accordance with Ethics committee University Hospital in Pilsen and approved experimental designs for appropriate experimental animal project.

In the study, we detected the dimethylation of histone H3 on lysine K4 (H3K4me2) as potential epigenetic marker of sperm quality and chromatin immaturity. Secondly, we observed the role of the gasotransmitter hydrogen sulphide (H_2S) as anti-capacitating agents, slowing down capacitation possibly through post-translational modification of proteins. Thirdly, SIRT1 histone deacetylase was estimated during oocytes maturation and its relocation from germinal vesicle of GV oocytes to the surround of meiotic spindle of metaphase II oocyte was observed. Subsequent part of the study is dealing with the effect of BPS on quality of gametes; malformation of meiotic spindle and aberration of dimethylation of histone H3 on lysine K27 (H3K27me2) in oocytes were observed. In male reproduction, BPS effect was mainly displayed by change in spectrum of acetylated proteins

To conclude, the study highlights importance of the epigenetic based marker of gametes, underlining spermatozoa, having potential to diagnose endocrine disruptor-derived reproduction failure. Accordingly, the knowledge can improve ART and increase embryo quality, through appropriate approach of sperm selection.

CONTENT

LIST OF ABBREVIATION

5meC	5-methylacytosine
ART	assisted reproductive therapy
BPA	bisphenol A
BPS	bisphenol S
CBS	cystathionine beta synthase
CTH	cystathionine gamma lyase
DFI	DNA fragmentation index
GV	germinal vesicle
GVBD	germinal vesicle breakdown
H2AK119ub	ubiqutination of histone H2A at lysine K119
H_2S	hydrogen sulfide
H3K27me2	dimethylation of histone H3 at lysine K27
H3K4me2	dimethylation of histone H3 at lysine K4
H3K9me3	trimethylation of histone H3 at lysine K9
HDS	high DNA stainability
LSD1	histone demethylase lysine-specific histone demethylase
MPST	3-mercaptopyruvate transferase
ncRNA	non-coding RNA
NO	nitric oxide
PGCs	primordial germ cells
PTM	post-translational modification
SCSA	sperm chromatin structure assay
SIRT1	sirtuin 1, NAD^+-dependent deacetylase
NaHS	natrium hydrosulfide monohydrate

1   INTRODUCTION

The formation of new life is preceded by fertilization, defined as fusion of gametes. Accordingly quality of oocytes and spermatozoa is inevitable factor predicting fertilization success and healthy embryo development[1]. Their formation, called gametogenesis already starts during embryonal development and is accompanied by the reduction of chromosomes to half, morphological changes as well as establishment of heritable epigenome typical for oocytes and spermatozoa[2–4].

Oocyte development, oogenesis is stopped two times during development. First meiotic block happened during prenatal age and its continue is restored in puberty. Subsequently, oocyte entries to second meiotic division, where is blocked until time of fertilization. Oocyte and sperm fusion is the impulse for oocyte reactivation and thus finishing the last division[2,5–8]. This is accompanied by various kinases and factors of ubiquitin-proteasome system. Moreover, dynamic epigenetic changes, including a lot upstream regulators, are required and many of them remain unelucidated[9–12].

In contrary spermatozoa are produced all reproductive life of men in the process called spermatogenesis without interruption[4,13,14]. Spermatozoa are unique due to their specific chromatin structure that preserves theirs DNA for embryo. During elongation of spermatid, apart from morphological changes involving formation of acrosome and flagellum, 85-90% of histones are replaced by protamines, that enable higher compaction of sperm chromatin[15–17]. However some residual histones undergoing posttranslational modifications persist and are usually related with genes involved in the early embryo development. Since ejaculated spermatozoa are transcriptionally inactive, epigenome established during spermatogenesis, is only thing that they can rely on[18–23].

This time of gamete development is known for its sensitivity to external factors coming from surrounds. Considering postponing parenthood, the gametes and their development are exposed to positive and negative factors much longer[24–27]. Hence, side by side with the rise of assisted reproductive therapy usage, information rise in this area was noticed too. Accordingly, current studies found out relation between ART usage and genetically imprinted disorder. Moreover gamete epigenome research reveals its impact on their quality and thus embryos[28–31]. Taking into account current massive using of ART bypassing inner molecular mechanisms is striking[32], and the necessity of studying the epigenetic impact and avoiding its harmful effect is crucial. Hence, a rise up of studies dealing with epigenetic-based sperm

selection markers in combination with non-invasive methods is not surprising. Most of them try to use principles of sperm selection in the female reproductive tract and use superficial sperm markers to avoid their damage[33–36].

2   HYPOTHESIS AND AIMS

Our attention is paid to epigenome of sperm and oocytes with the aim find our appropriate marker of gametes quality with potential to improve ART (Fig. 1).

Hypothesis:

1. Spermatozoa with immature chromatin express more H3K4me2, and thus it is adequate marker of spermatozoa quality.
2. H_2S acts as signal molecule in sperm physiology.
3. SIRT1 acts as signal molecule during oocyte maturation and through its epigenetic and non-epigenetic targets improve oocytes quality.
4. Endocrine disruptor bisphenol S (BPS) negatively affects gametogenesis and gametes quality.

The aims were as follows:

- To detect H3K4me2 in sperm with different quality, using flow cytometry strategy.
- To observe H_2S-releasing enzymes in sperm and describe the effect of exogenous H_2S supplementation on sperm quality.
- To track SIRT1 and its targets across the oocyte maturation.
- To investigate the effect of BPS on oocytes and sperm, as well as ovaries and testes, through selected markers.
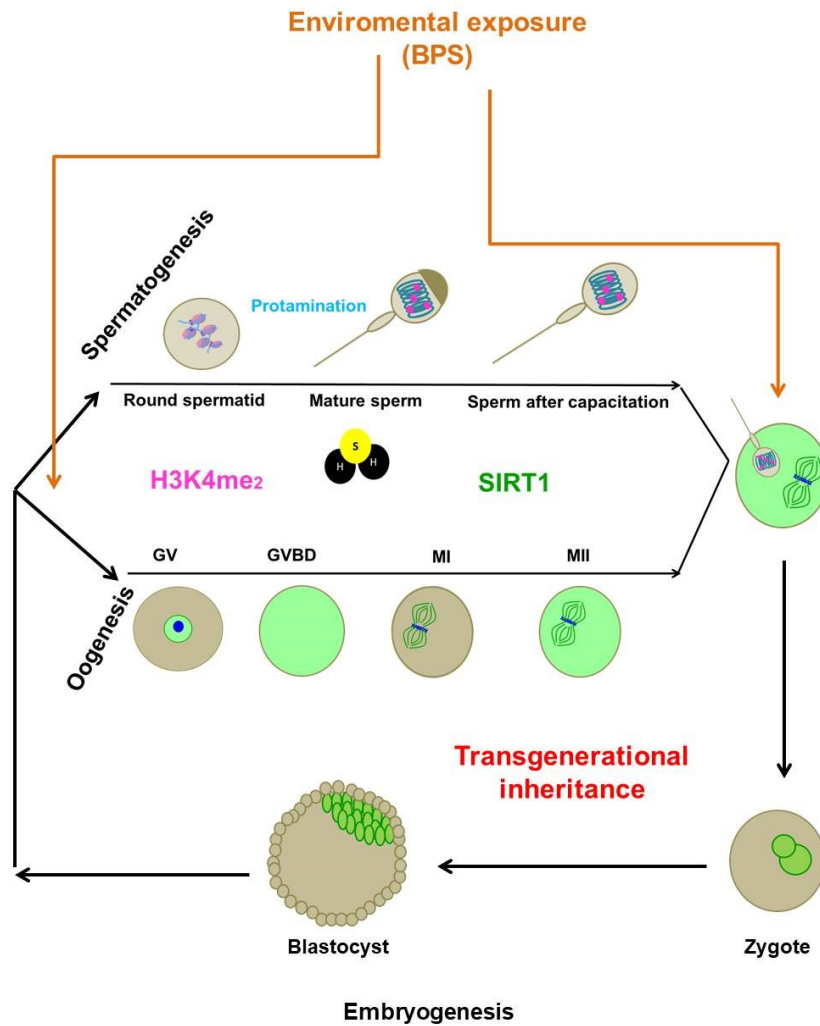
Figure 1. **Diagram describing hypothesis**. This diagram displays the most important events of gamete development, leading to fertilization. On the one side it is protamination through the spermatogenesis that also involves H3K4me2. If the protamination is not completed, ejaculated spermatozoa displays decrease quality as well as excess of H3K4me2. This event could be indirectly regulated by SIRT1. We consider SIRT1 to be versatile regulator of maturation of gametes, including oocytes. However, spermatozoa prior to fertilization have to undergo capacitation, this is possibly regulated by gasotansmitter H_2S through posttranslational modifications of proteins involved in that process. Sperm and oocytes development is accompanied by epigenetic changes that create exact program for embryo development. All these changes are inherited and sensitive to environmental exposure coming from surrounding daily life.

8

3 MATERIAL AND METHODS

3.1 Human sperm collection

Human ejaculates were obtained with the participants' written consent from the ART center Genetika Pilsen Ltd. (Pilsen, Czech Republic). All subjects were strictly anonymous to the research team. The study was approved by the Ethics committee of Charles University, Faculty of Medicine in Pilsen (238/2016). Altogether, 99 semen samples were collected. The evaluation of semen concentration and motility were carried out in accordance with the World Health Organization standards (WHO 2010). Accordingly, semen samples were classified based on semen quality into three groups: normozoospermic, asthenozoospermic and oligoasthenozoospermic.

3.2 Mice sperm and oocyte isolation

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. ICR mice were purchased from Velaz Ltd. (Prague, Czech Republic), housed in intact polysulfonate cages and maintained in a facility with a 12 L:12 D photoperiod, a temperature of 21±1 °C and a relative humidity of 60 %, and a phyto-estrogen-free diet 1814P (Altromin) and ultrapure water (in glass bottles, changed twice per week) were provided *ad libitum*.

The BPS experiment was designed to evaluate the effect of very low doses of BPS on the male reproductive system of 8 weeks old mice. Mice were randomly distributed to the four experimental groups and left to adapt for one week. BPS was then administered for eight weeks through drinking water. The exposure consisted of four BPS dose treatments: 0, 0.001, 1 and 100 $ng.g^{-1}$ bw. day^{-1}, i.e. vehicle control (VC) and BPS1 – BPS3 groups, respectively. In the female experiments, the exposure composed of five BPS dose treatments: 0, 0.001, 1, 10, 100 $ng.g^{-1}$ bw. day^{-1}, i.e. vehicle control (VC) and BPS1 – BPS4 groups.

Spermatozoa were isolated from both cauda epididymis and seminiferous tubules into 500 µl Whitten-HEPES medium and left to swim up for 10 minutes at 37 °C. Concentration and subjective motility was estimated using the Makler chamber and expressed as a percentage of motile spermatozoa.

For immature mouse oocyte yielding, ICR females were administered with i.p. 5 IU PMSG and sacrificed 48 hours later. Oocytes at the GV stage were isolated and *in vitro* matured in M16 medium for 16 h at 37°C and 5% CO_2. To obtain *in vivo* mature oocytes, PMSG-treated females were administered with 5 IU hCG, and cumulus-oocyte complexes were flushed from oviducts 16 hours later. Both *in vitro* and *in vivo* matured oocytes in metaphase II stage were used for further experiments.

3.3 Boar sperm collection

Fresh boar semen collections were completed under the strict guidance of an Animal Care and Use protocol approved by the Animal Care and Use Committee (ACUC) of the University of Missouri.

Fresh boar semen was collected on a weekly basis from three fertile boars used for routine. Concentration and motility of ejaculates were evaluated by conventional spermatological methods under a light microscope. Sperm concentration was measured by hemocytometer and ranged from 250 to 350 million/ml. Only ejaculates with >80% motility were used. Sperm rich fraction was used for all analysis, except analysis during in vitro capacitation (IVC).

3.4 Flow cytometry analysis

For the detection of mitochondrial activity and early apoptotic spermatozoa, sperm samples were incubated 30 minutes at room temperature with the staining solution of 100 nM MitoTracker Deep Red and 50nM YO-PRO1 in Biggers-Whitten Whittingham medium (BWW)[37]. Acquisition was performed on a FACSVerseTM flow cytometer using BD FACSuite™ software and analysed with WEASEL software Ver.3.

Sperm chromatin structure assay was performed according to the protocol described by Evenson and Jost[38]. The output of the analysis is DNA fragmentation index (%DFI) and high DNA stainability (%HDS). The samples were run by FACSVerse[TM] flow cytometer, controlled with BD FACSuite™ software and analysed with WEASEL software Ver.3 According to the results, samples were divided based of %HDS into two groups: low HDS – %HDS≤15, and high HDS – %HDS>15.

Preparation of spermatozoa for histone analysis by flow cytometry was done according to the study of Li [39]. For the detection of H3K4me2, polyclonal rabbit anti-H3K4me2 (1:100) were used, and Alexa Fluor 488 conjugated goat anti-rabbit secondary antibody (1:200). Acquisition was performed on a FACSVerse™ flow cytometer using BD FACSuite™ software and analysed with WEASEL software Ver 3.

3.5 Western blot analysis of sperm and oocytes

In the samples either cells or tissue, western blotting was performed. Samples were solubilized with the Laemmli buffer (0.003% Triton-X-100 and 0.001% SDS) and subjected to SDS-PAGE electrophoresis in precast gradient gels (4–12.5%), followed by blotting onto a PVDF membrane. The membrane was blocked in 1% BSA in TBS with 0.5% Tween-20 (TBS-T) and incubated with different antibodies rabbit polyclonal anti-H3K4me2 (1:1000), mouse monoclonal anti-SIRT1 (1:1000) and mouse polyclonal antibody anti α-tubulin (1:1000) overnight at 4°C. Then, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG in TBS-T (1:10000) for 1 h at room temperature. Proteins with adequate molecular weight were detected using the ECL Select Western Blotting Detection Reagent and visualised by ChemiDoc™ MP System.

3.6 Immunocytochemistry, microscopy and image analysis

Sperm samples were allowed to dry and fixed with 4% of paraformaldehyde for 15 minutes. Oocytes were fixed in 4% paraformaldehyde in PBS with 0.1% polyvinyl-alcohol (PVA) for 30 min. Samples were permeabilized and blocked for 1 hour at room temperature. The 1h incubation of samples with antibodies occured: anti-SIRT1 (1:200), anti-α tubulin (1:200), anti-acetylated α-tubulin (1:200), anti-H3K9me2/3 (1:200), anti-H3K4me2 (1:200), and anti-H2AK119ub (1:200), anti H3K27me2 (1:200) and anti-5′-methyl cytosine (1:200) was performed. Thereafter, samples were incubated with AlexaFluor conjugated goat anti-rabbit secondary antibodies (1:200). Finally, samples were mounted onto slides in a VectaShield medium with 4'6'-diamidino-2-phenylindole (DAPI). Images were acquired using spinning disk confocal microscope Olympus IX83 and VisiView® software.

3.7 Quantification of H₂S-releasing enzymes

Ejaculated and capacitated spermatozoa were compared for the expression of H_2S releasing enzymes. Sperm *in vitro* capacitation vas done as described below. Ejaculated and capacitated spermatozoa were washed for removing seminal plasma and capacitation medium, respectively. Subsequently spermatozoa were fixed with a cold acetone solution (1:1) in PBS, washed and blocked with 0.1% Triton TX-100 and 5% NGS for 40 minutes. Afterward, spermatozoa were incubated with primary antibody rabbit polyclonal anti-CBS (1:100), anti-CTH (1:100), anti-MPST (1:50) at 4 °C during the night. After two washings, incubation with secondary antibody Alexa fluor 488 (1:200), Hoechst 33342 (1:1500) and lectin PNA from Arachis hypogaea conjugated with Alexa fluor 647 (1:2500, PNA-AF647) was done for 40 minutes. The spermatozoa were then washed, and signal intensity was quantified using Amnis flow cytometry.

3.8 H₂S donor treatment of ejaculate spermatozoa and *in vitro* capacitation

Semen samples were washed of seminal plasma with non-capacitating media (NCM), a modified TL-HEPES medium, free of calcium dichloride ($CaCl_2$) and addition of 11mM D-glucose, with pH adjusted to 7.2. They were subsequently incubated with natrium hydrosulfide monohydrate, NaHS (2.5; 5; 10; 20; 100 μM) for 1 hour. Distilled water was used as vehicle control (VC). After 1 hour, the samples were incubated with cocktail of Hoechst 33342 (1:1000), PNA-AF647 (1:2000), Propidium Iodide (1:1000) and FZ3 (1:1500) for 30 minutes.

Subsequently, an aliquot of semen sample was resuspended in 0.5 mL *in vitro* capacitation media (IVC media), TL-HEPES-PVA supplemented with 5mM sodium pyruvate, 11mM D-glucose, 2mM $CaCl_2$, 2mM sodium bicarbonate, and 2% (m/v) bovine serum albumin, and incubated in a 37 °C water bath for 4 h, with Eppendorf tube rotation performed every 60 min. For studying the H_2S effect on sperm capacitation, the medium was supplemented with the selected concentration of H_2S donor NaHS. The capacitation status was measured each hour using Amnis flow cytometry with the same fluorescent probes and aforementioned protocol for ejaculated spermatozoa.

3.9 Acquisition of Amnis Flow cytometry

Ejaculated spermatozoa treated with H2S donor were measured after 1 hour of treatment with H_2S, and the effect was estimated by evaluation of membrane remodelling (PI), acrosomal status (PNA) and Zinc signature using Amnis Flow cytometry. Similarly, capacitation status in condition of H_2S treatment was observed and, during 4 hours of in vitro capacitation, all of the above mentioned parameters were measured hourly.

The acquisition for H_2S-releasing enzymes in ejaculated and capacitated spermatozoa as well as observation of H_2S donors on capacitation were carried out by INSPIRE® FS software.

3.10 Statistical analysis

Data from all analyses was expressed either as the medians with appropriate quantiles or means ± SEM. Data was processed with Statistica Cz 12 (StatSoft, USA), using Kruskal-Wallis ANOVA (for quantitative variables) or One-way ANOVA. With regard to the significant overall finding, differences between individual group pairs were assessed post-hoc using multiple comparisons of mean ranks and the Fisher test, respectively. In addition, selected parameters were subjected to Spearman's rank correlation test. The level of statistical significance was set at $p \leq 0.05$.

4 RESULTS

4.1 Sperm epigenetics is associated with sperm quality and maturity

Ninety-nine samples were characterized by the following semen parameters: age, volume, sperm concentration, total and progressive motility, mitochondrial activity/early apoptotic cells, DNA fragmentation (%DFI), chromatin immaturity (%HDS) and the level of H3K4me2. According to the concentration and motility in accordance with WHO standards 2010, semen samples were divided into three groups; normozoospermic (N), asthenozoospermic (A) and oligoasthenozoospermic (OA), and compared between themselves in assessed parameters. To characterize samples with different chromatin maturity, samples were divided into groups with low %HDS (HDS≤15) and high %HDS (HDS>15) that was assessed by SCSA® as referred Evenson and Jost.[40]

Chromatin integrity of the cohort was characterized by SCSA®, assessing both %DFI and %HDS [40]. Samples with a pathological spermiogram were exhibited significantly higher percentage of spermatozoa with DNA damage (%DFI). Despite the significant %HDS dependency on spermiogram quality, no significant differences were confirmed between individual groups. However, a significant trend (Spearman's correlation: $p=0.021$) of increasing %HDS was proven for decreasing level of spermiogram quality (N > A > OA). Moreover, the %HDS correlated positively with %DFI and negatively with sperm concentration, and total and progressive motility. We also found a negative correlation between the percentage of spermatozoa with active mitochondria (MitoTracker+/YO-PRO1-) and %HDS.

Finally, we observed that OA and A samples had higher H3K4me2 levels compared to samples with a normal spermiogram (N). These differences were statistically significant for OA and A samples compared to N ones. Similarly, significantly higher H3K4me2 levels were detected in the group with %HDS>15. Additionally, H3K4me2 levels were correlated positively with %HDS, but negatively with sperm concentration, progressive motility.

4.2   The involvement of H₂S in sperm capacitation

The study carries out the regulation of spermatozoa capacitation by H_2S. The preliminary data related to that, were achieved during fellow at University of Missouri, with kind help and supervision of prof. Peter Sutovsky, Dr.h.c. Recently; the data are under preparation for manuscript submission to a journal with impact factor.

We have detected all three H_2S-releasing enzymes in ejaculated and capacitated spermatozoa. In addition, we have quantified and compared these enzymes by Amnis flow cytometry. Our results show that signal intensity of all enzymes is decreased in capacitated spermatozoa in comparison to ejaculated, however only in the case of enzymes, cystahionine β-synthase, cystathionine ϒ-lyase, was this difference significant.

Secondly, we have observed that NaHS, an exogenous H_2S donors, had a sperm protective effect, what is displayed by percentage of propidium iodide positive cells, acrosome integrity (PNA). Moreover, H_2S donors were able to preserve zinc signature 1, as the sign of higher sperm fertility ability.

Finally, we have noticed capacitation slow down under condition of H_2S, displayed by preserve membrane and acrosomal integrity as well as zinc signature 1.

4.3 SIRT1 histone deacetylase plays a role in histone code establishment in oocyte

In this study, we have described how SIRT1 is involved in oocyte maturation through a description of new cytoskeletal SIRT1 targets implicated in an oocyte division. Relocation of SIRT1 from the germinal vesicle of GV oocytes to the meiotic spindle of metaphase II oocytes pointed out intentional SIRT1 displacement to deacetylase tubulin of spindle microtubules (Fig. 14). This finding was also supported by the observation of specific histone PTMs that are clearly related to SIRT1 action, such as an increase in H3K9me3 and H2AK119ub as heterochromatin markers and a decrease in H3K4me2 associated with active transcription. Therefore, two molecular matters of SIRT1 action oocytes are considered: epigenetic and non-epigenetic. The fast exchange of both modes is obvious, and this is a topic for further study, including male germ cells and spermatozoa.

Altogether, important protecting and stabilizing functions of SIRT1 for oocytes chromatin were highlighted, as well as for their proper maturation to give rise to a healthy embryo. Furthermore, relocation of SIRT1 to the meiotic spindle surround may be relevant for the early post-fertilization events.

4.4 The environmental impact on fertility through spermatogenesis, oogenesis

Based on previously achieved knowledge of gamete biology, this study investigated endocrine disruptive effect of bisphenol S (BPS), a common environmental pollutant, on gamete quality.

The ability of endocrine disruptors to interfere with hormones is displayed by the defect during gametogenesis[41–43]. Our studies have shown decrease in antral follicles after BPS treatment, which point to the obliteration of oogenesis. Furthermore, the ability of the ovaries to protect themselves and inner ongoing oogenesis against oxidative stress were proven, which is evident from the decreased expression of superoxide dismutase (SOD). The effect of BPS was also displayed by the aberration of oocytes epigenetic, manifested by H3K27me2 and related to gene imprinting, and thus possible defects in further embryo development. Further, defects in DNA methylation were assessed as 5meC (5-methylcytosine). Moreover, BPS increase DNA fragmentation and disrupt DNA methylation. The striking detrimental effect of BPS was also investigated on the meiotic spindle, which is responsible for correct chromosomes organization and subsequent redistribution that may have an effect on oocyte maturation, as well as embryo development.

In the case of male reproduction, published studies showed that BPS influence the testosterone level and disrupt oxidative stress defence[41]. A decrease in sperm concentration and motility were also affected by BPS[44]. This is in accordance with our study, where we observed the decrease in motility in one BPS concentration. Nevertheless, we have not found significant decreases in male fertility manifested by an aberration of hormone production or histology observations. Proteomic analysis of mice testes showed that used subliminal doses of BPS influence proteosynthesis and posttranslational modifications of proteins, manifested by exclusive expression of 34 proteins in the testes of BPS treated mice, and 20 other proteins were specifically acetylated in BPS groups.

Accordingly, BPS seems to affect the protein's functionality; however, subsequent observation is crucial to understanding if acetylation of proteins increase their activity as in the case of histones, or if it disables their functionality. Interestingly, our results showed that BPS affects mainly proteins associated with sperm motility and capacitation.

All of the data showed that endocrine disruptors are silent poisoners of our reproductive health, influencing the functionality of the cells on the epigenetic level and, by interfering with hormones, obstruct cells in their defence. Since the endocrine disruptive effect is displayed in the histone code of gametes, the effect on early embryo development is plausible. Hence, these studies should be a base for preventive action, starting with an estimation of BPS intake in the population, as well as more conscious observation of potential safety substituents involving appropriate epigenetic based markers.

4.5   The implications of sperm epigenetic quality in human ART

Nowadays, together with an increasing number of couples with conceiving problems, assisted reproductive therapy (ART) has significantly raised too. Therefore, the investigation of the reasons hidden behind this phenomenon, as well as the improvement of screening methods in ART is current. Apart from this, studies associating infertility with defects in epigenetic code of either oocytes or sperm are being released on a daily basis[23,45–48]. Hence, it is more desirable to consider the area of epigenetic in ART. Currently, there are already well established epigenetic based markers that have the potential to be used either in gamete screening, and thus the establishment of appropriate treatment, or their selection for ART[34,49,50]. In the case of sperm selection, apart from the well-defined markers, use of the non-invasive method is crucial.

16

In this review, we have offered an outlook on sperm selection methods that may imply epigenetic based markers such as ɣH2AX or H3K4me2 described by us. The trend in this area is to mimic the selection mechanism of female genital tract as much as possible and thus approximate to natural conditions. Our attention was paid to three non-invasive methods that have the potential to apply this, namely microfluidic, Raman spectrometry and sperm ubiquitin taq assay. However, further investigation is necessary for confirmation of their harmless effect, as well as the estimation of the best method for their clinical application.

5   DISCUSSION

Gamete epigenetics is still underestimated study object in assisted reproductive therapy (ART), and thus we consider it inevitable to investigate gamete epigenome and look for possible markers of gamete quality that will have the potential to be used for either gamete screening or selection for ART.

Since ejaculated spermatozoa are not capable of protein expression, they have to rely on the resources obtained during their development[51,52]. However, spermatogenesis is quite sensitive to oxidative stress[53–56], and thus we can find in ejaculates a various subpopulation of sperm of different quality. Our results clearly declared that spermatozoa with decreased quality and incomplete chromatin condensation have increase expression of H3K4me2. Considering the position of H3K4me2 at the promotors of housekeeping genes, as well as genes for small noncoding RNA[19,57–60], it is plausible that H3K4me2 overexpression may effect in early post-fertilization events and embryo development. Furthermore, taking into account the higher expression of H3K4me2 in the early stages of spermatogenesis prior to protamination, observed by Rathke *et al.*[58], we consider H3K4me2 a relevant indicator of spermatozoa quality and chromatin integrity. The results highlighted the importance of involving epigenetics-based markers in the clinical practise of ART. The attitude to its application could be various; on the one hand it could serve as a screening parameter for the verification of sperm quality after routinely used sperm selection techniques. On the other hand, in combination with non-invasive methods such as Raman spectrometry, it could be used as a direct selection marker, and thus sperm used in ART. Apart from this, we consider it really important to understand why the mistakes in protamination happened and finding ways to avoid them. For this purpose, we need to understand all of the pathways involved in spermatogenesis.

However, the best way to increase sperm fertilization success is to improve their quality and protect spermatozoa through spermatogenesis. Hence, it is indispensable to understand the pathways involved in H3K4me2 demethylation and methylation. Interestingly, many histone deacetylases are capable of modulating even the methylation of histones and, therefore, this protein family deserves our interest[61–63]. Accordingly, one of the possible regulators of histone methylation is SIRT1, a member of III class NAD^+-dependent histone deacetylases, called sirtuins[64,65]. Indeed, SIRT1 participates in the regulation of H3K4me2 through histone demethylase lysine-specific histone demethylase 1A (LSD1A). Our study of SIRT1 during oocytes maturation, declared the relocation of SIRT1 from germinal vesicle in GV oocytes to cytoplasm of metaphase II oocytes that are supposed to be ready for fertilization. This finding confirmed the protective role of SIRT1 for oocytes chromatin and observed different targets of SIRT1 that are deacetylated according to oocytes requirements. Furthermore, colocalization of SIRT1 with tubulin of meiotic spindle in oocyte cytoplasm uncovered a huge spectrum of possibilities that may explain the SIRT1 relocation. Since mature spermatozoa have just a subliminal amount of SIRT1, we can speculate that SIRT1 in the cytoplasm of metaphase II oocyte is indispensable for fertilization and mainly early post-fertilization events, including mitophagy, chromosome organisation through sperm aster and pronuclei formation, and thus a compensate lack of SIRT1 in sperm

All these events could be regulated by gasotransmitters (hydrogen sulphide, nitric oxide), gaseous signal molecule that may influence the cell physiology through PTMs of proteins[66]. Considering the involvement of nitric oxide in the capacitation through nitrosylation of various proteins[67], we have looked at the expression, and thus role of H_2S during capacitation. Our results proved that capacitation is inhibited under conditions of H_2S donors. This finding is in accordance with the study by Wang *et al.*[53], who observed reversibility of hyperactivated movement by H_2S donors. The presumed mechanism is through sulfhydration of proteins involved in the capacitation, and thus further studies should be focused in this direction. Moreover, the description of SIRT1 upregulation by its sulfhydration changes the view at the epigenome regulation[68]. Hence, gasotransmitters are potential molecules participating in sperm physiology and possibly also in the histone code. Understanding of their mechanism of action can offer either new markers of gamete quality or infertility therapy.

The aforementioned factors can be considered physiological modulators of the epigenetic code. On the other hand, environmental influences are able to modulate as well, and the risk of various noxi arises[27,69–72]. In addition to sperm histone code and gasotransmission through hydrogen sulphide, we were focused on selected pollutants for model of environmental-derived modulation of sperm physiological status, including epigenetic factors. Therefore, we have interpolated our knowledges about epigenetic of gametes into the observation endocrine disruptive effect of bisphenol S (BPS), one of most used environmental pollutants[9,73–75], on gamete quality. Our finding declared that subliminal doses of BPS that are much less than a tolerable daily intake of BPA have negative effect on gametogenesis displayed by aberration either in epigenome or general proteosynthesis by affecting their PTMs. Apart from this, the negative effect of BPS was displayed by the malformation of meiotic spindle crucial for correct chromosome redistribution during oocytes maturation, as well as for subsequent embryo development. This finding highlights that replacement of BPA by BPS was not the best solution, and sufficient declaration of the harmless effect on reproductive health of individuals before it was done were missing. Hence, we consider indispensable estimates of the daily intake of BPS in the human population and accordingly adapt other steps for health protection, such as replacement of BPS by a compound that will be declared as harmless on different cellular and molecular levels, including epigenetic.

Altogether, the study highlights the consideration of the epigenome in the evaluation of gamete quality. However, the base for this is to understand epigenetic regulation and the consequences for embryo development. Therefore, further studies should be focused on finding the best way to combine currently used non-invasive methods and selected epigenetic markers. Considering epigenome aberration as a plausible reason of idiopathic infertility, selected markers can be used for the evaluation of gamete quality and, accordingly, the establishment of the correct therapy, or they could serve as selection markers for a healthier gamete. We expect that the increase application in epigenetic based methods in ART will improve fertilization success and embryo quality and will be prevention to epigenome based disorders in offspring.

6   CONCLUSION

The aim of study was investigation of new epigenetics-based markers in gametes that have potential to be applied in ART either for gametes screening or their selection for further therapy.

We have estimated new epigenome based sperm selection marker H3K4me2 which expression is dependent on spermatozoa quality and chromatin immaturity. This histone modification is indirectly regulated by SIRT1, participating in the protamination too. Accordingly, epigenetic and non-epigenetic targets of SIRT1 in oocytes have been identified, offering the possibility of their implication during early post-fertilization events in embryo. Furthermore, the role of gasotransmitter H_2S has been observed as the new signalling molecule in sperm physiology, that slow down capacitation possibly through posttranslational modifications of proteins involved in that process. Finally, endocrine disruptive effect of BPS was confirmed on sperm and oocytes through modification of histone code as well as other PTMs of proteins.

Altogether, epigenetic markers, sensitive to environmental exposure, are a promising indicator of gametes quality and a predictor of fertilization success. Accordingly, we consider as exigency to update non-invasive sperm selection methods with epigenetic based marker and thus improve ART.

REFERENCES

1. Kushnir, V. A., Barad, D. H., Albertini, D. F., Darmon, S. K. & Gleicher, N. Systematic review of worldwide trends in assisted reproductive technology 2004–2013. *Reprod. Biol. Endocrinol.* **15**, 6 (2017).

2. De Felici, M. Origin, Migration, and Proliferation of Human Primordial Germ Cells. in *Oogenesis* 19–37 (Springer London, 2013). doi:10.1007/978-0-85729-826-3_2

3. Gosden, R. G. Oogenesis as a foundation for embryogenesis. *Mol. Cell. Endocrinol.* **186**, 149–53 (2002).

4. Griswold, M. D. Spermatogenesis: The Commitment to Meiosis. *Physiol. Rev.* **96**, 1 (2016).

5. Mehlmann, L. M. Signaling for Meiotic Resumption in Granulosa Cells, Cumulus Cells, and Oocyte. in *Oogenesis* 171–182 (Springer London, 2013). doi:10.1007/978-0-85729-826-3_12

6. Detlaf, T. A., Vassetzky, S. G. & Billett, F. *Oocyte Growth and Maturation*. (Springer US, 1988). doi:10.1007/978-1-4684-0682-5

7. Demirtas, E., Holzer, H., SON, W.-Y., Chain, R.-C. & Tan, S. L. In-vitro Maturation of Human Oocytes. in *Reproductive Endocrinology and Infertility* 633–646 (Springer New York, 2010). doi:10.1007/978-1-4419-1436-1_42

8. Tripathi, A., Prem Kumar, K. V. & Chaube, S. K. Meiotic cell cycle arrest in mammalian oocytes. *J. Cell. Physiol.* **223**, 592–600 (2010).

9. Adamkova, K. *et al.* SIRT1-dependent modulation of methylation and acetylation of histone H3 on lysine 9 (H3K9) in the zygotic pronuclei improves porcine embryo development. *J. Anim. Sci. Biotechnol.* **8**, 83 (2017).

10. Dean, W. Epigenetic Regulation of Oocyte Function and Developmental Potential. in *Oogenesis* 151–167 (Springer London, 2013). doi:10.1007/978-0-85729-826-3_11

11. Zuccotti, M., Merico, V., Redi, C. A. & Garagna, S. An Epigenomic Biography of the Mammalian Oocyte. in *Oogenesis* 141–150 (Springer London, 2013). doi:10.1007/978-0-85729-826-3_10

12. Luciano, A. M. & Lodde, V. Changes of Large-Scale Chromatin Configuration During Mammalian Oocyte Differentiation. in *Oogenesis* 93–108 (Springer London, 2013). doi:10.1007/978-0-85729-826-3_7

13. Holstein, A.-F., Schulze, W. & Davidoff, M. Understanding spermatogenesis is a prerequisite for treatment. *Reprod. Biol. Endocrinol.* **1**, 107 (2003).

14. *Molecular Mechanisms in Spermatogenesis.* **636**, (Springer New York, 2008).

15. Steger, K. & Balhorn, R. Sperm nuclear protamines: A checkpoint to control sperm chromatin quality. *Anat. Histol. Embryol.* **47**, 273–279 (2018).

16. Oliva, R. & Ballescà, J. L. Altered histone retention and epigenetic modifications in the sperm of infertile men. *Asian J. Androl.* **14**, 239–240 (2012).

17. Soler-Ventura, A. *et al.* Mammalian Sperm Protamine Extraction and Analysis: A Step-By-Step Detailed Protocol and Brief Review of Protamine Alterations. *Protein Pept. Lett.* **25**, 424–433 (2018).

18. Rodgers, A. B., Morgan, C. P., Leu, N. A. & Bale, T. L. Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. *Proc. Natl. Acad. Sci.* **112**, 13699–13704 (2015).

19. Hammoud, S. S. *et al.* Distinctive chromatin in human sperm packages genes for embryo development. *Nature* **460**, 473–478 (2009).

20. Siklenka, K. *et al.* Disruption of histone methylation in developing sperm impairs offspring health transgenerationally. *Science (80-.).* **350**, aab2006–aab2006 (2015).

21. Lambrot, R., Siklenka, K., Lafleur, C. & Kimmins, S. The Genomic Distribution of Histone H3K4me2 in Spermatogonia is Highly Conserved in Sperm. *Biol. Reprod.* (2019). doi:10.1093/biolre/ioz055

22. Schon, S. B. *et al.* Histone modification signatures in human sperm distinguish clinical abnormalities. *J. Assist. Reprod. Genet.* **36**, 267–275 (2019).

23. Kutchy, N. A. *et al.* Acetylation and methylation of sperm histone 3 lysine 27 (H3K27ac and H3K27me3) are associated with bull fertility. *Andrologia* e12915 (2017). doi:10.1111/and.12915

24. Buser, M. C., Abadin, H. G., Irwin, J. L. & Pohl, H. R. Windows of sensitivity to toxic chemicals in the development of reproductive effects: an analysis of ATSDR's toxicological profile database. *Int. J. Environ. Health Res.* **28**, 553–578 (2018).

25. Luderer, U. Ovarian Toxicity from Reactive Oxygen Species. in *Vitamins and hormones* **94**, 99–127 (2014).

26. Schuh-Huerta, S. M. *et al.* Genetic variants and environmental factors associated with hormonal markers of ovarian reserve in Caucasian and African American women.

Hum. Reprod. **27**, 594–608 (2012).

27. Estill, M. S. & Krawetz, S. A. The Epigenetic Consequences of Paternal Exposure to Environmental Contaminants and Reproductive Toxicants. *Curr. Environ. Heal. Reports* **3**, 202–213 (2016).

28. Tang, L. *et al.* Imprinting alterations in sperm may not significantly influence ART outcomes and imprinting patterns in the cord blood of offspring. *PLoS One* **12**, e0187869 (2017).

29. Yetunde, I. & Vasiliki, M. Effects of advanced selection methods on sperm quality and ART outcome. *Minerva Ginecol.* **65**, 487–496 (2013).

30. Belva, F. *et al.* Semen quality of young adult ICSI offspring: the first results. *Hum. Reprod.* **31**, 2811–2820 (2016).

31. Mani, S. & Mainigi, M. Embryo Culture Conditions and the Epigenome. *Semin. Reprod. Med.* **36**, 211–220 (2018).

32. Suarez, S. S. Mammalian sperm interactions with the female reproductive tract. *Cell Tissue Res.* **363**, 185–194 (2016).

33. Eskandari-Shahraki, M., Tavalaee, M., Deemeh, M. R., Jelodar, G. A. & Nasr-Esfahani, M. H. Proper ubiquitination effect on the fertilisation outcome post-ICSI. *Andrologia* **45**, 204–210 (2013).

34. Ozanon, C., Chouteau, J. & Sutovsky, P. Clinical adaptation of the sperm ubuquitin tag immunoassay (SUTI): relationship of sperm ubiquitylation with sperm quality in gradient-purified semen samples from 93 men from a general infertility clinic population. *Hum. Reprod.* **20**, 2271–2278 (2005).

35. Liu, Y., Zhu, Y. & Li, Z. Application of Raman spectroscopy in Andrology: non-invasive analysis of tissue and single cell. *Transl. Androl. Urol.* **3**, 125–33 (2014).

36. Smith, G. D. & Takayama, S. Application of microfluidic technologies to human assisted reproduction. *Mol. Hum. Reprod.* **23**, gaw076 (2017).

37. Biggers, J. D., Whitten, W. K. & Whittingham, D. G. The culture of mouse embryos in vitro. *Cult. mouse embryos Vitr.* (1971).

38. Evenson, D. & Jost, L. Sperm Chromatin Structure Assay for Fertility Assessment. in *Current Protocols in Cytometry* (John Wiley & Sons, Inc., 2001). doi:10.1002/0471142956.cy0713s13

39. Li, Z., Yang, J. & Huang, H. Oxidative stress induces H2AX phosphorylation in human

spermatozoa. *FEBS Lett.* **580**, 6161–6168 (2006).

40. Evenson, D. & Jost, L. Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell Sci.* **22**, 169–89 (2000).

41. Ullah, A. *et al.* Bisphenol A and its analogs bisphenol B, bisphenol F, and bisphenol S: Comparative in vitro and in vivo studies on the sperms and testicular tissues of rats. *Chemosphere* **209**, 508–516 (2018).

42. Rouiller-Fabre, V., Habert, R. & Livera, G. Effects of endocrine disruptors on the human fetal testis. *Ann. Endocrinol. (Paris).* **75**, 54–57 (2014).

43. Cox, K. H., Gatewood, J. D., Howeth, C. & Rissman, E. F. Gestational exposure to bisphenol A and cross-fostering affect behaviors in juvenile mice. *Horm. Behav.* (2010). doi:10.1016/j.yhbeh.2010.07.008

44. Shi, M., Sekulovski, N., MacLean, J. A. & Hayashi, K. Effects of bisphenol A analogues on reproductive functions in mice. *Reprod. Toxicol.* (2017). doi:10.1016/j.reprotox.2017.06.134

45. Jenkins, T. G. & Carrell, D. T. The sperm epigenome and potential implications for the developing embryo. *Reproduction* **143**, 727–734 (2012).

46. Tavalaee, M., Razavi, S. & Nasr-Esfahani, M. H. Influence of sperm chromatin anomalies on assisted reproductive technology outcome. *Fertil. Steril.* **91**, 1119–1126 (2009).

47. Ge, Z.-J., Schatten, H., Zhang, C.-L. & Sun, Q.-Y. Oocyte ageing and epigenetics. *Reproduction***149**, R103–R114 (2015).

48. Osman, E., Franasiak, J. & Scott, R. Oocyte and Embryo Manipulation and Epigenetics. *Semin. Reprod. Med.* **36**, e1–e9 (2018).

49. Zhong, H. *et al.* Evaluating γH2AX in spermatozoa from male infertility patients. *Fertil. Steril.* **104**, 574–581 (2015).

50. Francis, S., Yelumalai, S., Jones, C. & Coward, K. Aberrant protamine content in sperm and consequential implications for infertility treatment. *Hum. Fertil. (Camb).* **17**, 80–9 (2014).

51. Jenkins, T. G. & Carrell, D. T. The paternal epigenome and embryogenesis: poising mechanisms for development. *Asian J. Androl.* **13**, 76–80 (2011).

52. Vigodner, M. *et al.* Localization and identification of sumoylated proteins in human sperm: Excessive sumoylation is a marker of defective spermatozoa. *Hum. Reprod.* **28**,

210–223 (2013).

53.   Wang, J. *et al.* Hydrogen Sulfide As a Potential Target in Preventing Spermatogenic Failure and Testicular Dysfunction. *Antioxid. Redox Signal.* **28**,(2018)

54.   Delbes, G., Hales, B. F. & Robaire, B. Toxicants and human sperm chromatin integrity. *Mol. Hum. Reprod.* **16**, 14–22 (2010).

55.   Kaur, S., Saluja, | M, Bansal, | M P & Kaur, C. S. Bisphenol A induced oxidative stress and apoptosis in mice testes: Modulation by selenium. *Andrologia* (2017). doi:10.1111/and.12834

56.   Gunes, S., Arslan, M. A., Hekim, G. N. T. & Asci, R. The role of epigenetics in idiopathic male infertility. *J. Assist. Reprod. Genet.* **33**, 553–569 (2016).

57.   Wilson, S. *et al.* Localization of dimethylated histone three lysine four in the Rattus norvegicus sperm genome. *Biol. Reprod.* **0**, 1–3 (2018).

58.   Rathke, C. *et al.* Transition from a nucleosome-based to a protamine-based chromatin configuration during spermiogenesis in Drosophila. *J. Cell Sci.* **120**, 1689–1700 (2007).

59.   Goodman, J. E. *et al.* Weight-of-Evidence Evaluation of Reproductive and Developmental Effects of Low Doses of Bisphenol A. *Crit. Rev. Toxicol.* **39**, 1–75 (2009).

60.   Brykczynska, U. *et al.* Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. *Nat. Struct. Mol. Biol.* **17**, 679–687 (2010).

61.   Mulligan, P. *et al.* A SIRT1-LSD1 Corepressor Complex Regulates Notch Target Gene Expression and Development. *Mol. Cell* **42**, 689–699 (2011).

62.   Yu, C.-W. *et al.* Histone deacetylase6 Acts in Concert with Histone Methyltransferases SUVH4, SUVH5, and SUVH6 to Regulate Transposon Silencing. *Plant Cell* **29**, 1970–1983 (2017).

63.   Tatone, C. *et al.* Sirtuin Functions in Female Fertility: Possible Role in Oxidative Stress and Aging. *Oxid. Med. Cell. Longev.* **2015**, 1–11 (2015).

64.   Vaquero, A. *et al.* SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation. *Nature* **450**, 440–444 (2007).

65.   Bosch-Presegué, L. & Vaquero, A. Sirtuin-dependent epigenetic regulation in the maintenance of genome integrity. *FEBS J.* **282**, 1745–1767 (2015).

66.   Nevoral, J., Bodart, J.-F. F. & Petr, J. Gasotransmitters in Gametogenesis and Early

Development: Holy Trinity for Assisted Reproductive Technology - A Review. *Oxid. Med. Cell. Longev.* **2016**, 1730750 (2016).

67. Lefièvre, L. *et al.* Human Spermatozoa Contain Multiple Targets for Protein S-Nitrosylation: An Alternative Mechanism of the Modulation of Sperm Function by Nitric Oxide? *Proteomics* **7**, 3066–84 (2007).

68. Du, C. *et al.* Sulfhydrated Sirtuin-1 Increasing Its Deacetylation Activity Is an Essential Epigenetics Mechanism of Anti-Atherogenesis by Hydrogen Sulfide. *Antioxid. Redox Signal.* ars.2017.7195 (2018). doi:10.1089/ars.2017.7195

69. Cabaton, N. J. *et al.* Perinatal exposure to environmentally relevant levels of bisphenol A decreases fertility and fecundity in CD-1 mice. *Environ. Health Perspect.* **119**, 547–552 (2011).

70. Xie, M. *et al.* Neonatal bisphenol A exposure induces meiotic arrest and apoptosis of spermatogenic cells. *Oncotarget* (2016). doi:10.18632/oncotarget.7218

71. Brieño-Enríquez, M. A. *et al.* Exposure to endocrine disruptor induces transgenerational epigenetic deregulation of microRNAs in primordial germ cells. *PLoS One* **10**, 1–19 (2015).

72. Vecoli, C., Montano, L. & Andreassi, M. G. Environmental pollutants: genetic damage and epigenetic changes in male germ cells. *Environ. Sci. Pollut. Res.* **23**, 23339–23348 (2016).

73. Ullah, A., Pirzada, M., Jahan, S., Ullah, H. & Khan, M. J. Bisphenol A analogues bisphenol B, bisphenol F, and bisphenol S induce oxidative stress, disrupt daily sperm production, and damage DNA in rat spermatozoa: a comparative in vitro and in vivo study. *Toxicol. Ind. Health* **35**, 294–303 (2019).

74. Nevoral, J. *et al.* Long-term exposure to very low doses of bisphenol S affects female reproduction. *Reproduction* **156**, 47–57 (2018).

75. Rosenmai, A. K. *et al.* Are structural analogues to bisphenol a safe alternatives? *Toxicol. Sci.* (2014). doi:10.1093/toxsci/kfu030

7   APPENDIX

A1

Štiavnická M, García-Álvarez O, Ulčová-Gallová Z, Sutovsky P, Abril-Parreño L, Dolejšová M, Řimnáčová H, Moravec J, Hošek P, Lošan P, Gold L, Fenclová T, Králíčková M, Nevoral J. The H3K4me2 accompanies the chromatin immaturity of 1 human spermatozoa: an epigenetic marker for sperm quality assessment. *Systems Biology in Reproductive Medicine*. 2019. Under review (**IF$_{2017}$=1.582**)

A2

Nevoral J, Landsmann L, **Štiavnická M**, Hošek P, Moravec J, Prokešová Š, Řimnáčová H , Koutná E, Klein P, Hošková K, Žalmanová T, Petr J, Králičková M. Epigenetic and non-epigenetic mode of SIRT1 action during oocyte meiosis progression. *Journal of Animal Science and Biotechnology*. 2019. In press (**IF$_{2017}$=3,205**)

A3

Nevoral J, Kolinko Y, Moravec J, Žalmanová T, Hošková K, Prokešová Š, Klein P, Ghaibour K, Hošek P, **Štiavnická M**, Řimnáčová H, Tonar Z, Petr J, Králíčková M. Long-term exposure to very low doses of bisphenol S affects female reproduction. *Reproduction.* 2018; 156(1):47-57. (**IF$_{2017}$=3,086**)

A4

Štiavnická M, Abril-Parreño L, Nevoral J, Králíčková M, García-Álvarez O. Non-Invasive Approaches to Epigenetic-Based Sperm Selection. 2017; *Medical science monitor*. 2017; 23:4677-4683. (**IF$_{2017}$=1,89**)