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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

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PREFACE

This work was made up at the Biomedical Center and Department of Histology and Embryology, Faculty of Medicine in Pilsen, Charles University, under the supervision of doc. Ing. Jan Nevoral, Ph.D. and DVM. Olga García-Álvarez, Ph.D.

I declare that I wrote this thesis by myself and all the literary resources are properly cited. The present work was not used to obtain another or the same scientific degree. The text of this thesis comprised the parts of the author´s scientific articles, whose full texts are available in the attachments to this work.

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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 the 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
ATM	ataxia-telangiectasia mutated
ATR	ataxia- and Rad3-related
BPA	bisphenol A
BPS	bisphenol S
BRDT	bromodomain testis associated proteins
CBS	cystathionine beta synthase
CO	carbon monooxide
CTH	cystathionine gamma lyase
DFI	DNA fragmentation index
DNA-PK	DNA-dependent protein kinase.
DNMT	DNA methyltransferase
GV	germinal vesicle
GVBD	germinal vesicle breakdown
H2AK119ub	ubiqutination of histone H2A at lysine 119
H_2S	hydrogen sulphide
H3K27me2	dimethylation of histone 3 at lysine K27
H3K4me2	dimethylation of histone 3 at lysine K4
H3K9me3	trimethylation of histone 3 at lysine K9
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDS	high DNA stainability
JHDM2A, JMJD1A,KDM3A	JmjC domain-containing histone demethylase 2A
lncRNA	long non-coding RNA
LSD1	histone demethylase lysine-specific histone demethylase 1A
MDC1	mediator of DNA damage checkpoint 1
miRNA	micro RNA
MPST	3 mercapto pyruvate transferase

MSCI	meiotic sex chromosome inactivation
MTOC	microtubule organizing center
NaHS	natrium hydrosulfide monohydrate
ncRNA	non-coding RNA
NO	nitric oxide
PAWP	post-acrosomal WW-domain binding protein
PGCs	primordial germ cells
PIKKs	phosphatidylinositol 3-kinase-related kinases
piRNA	piwi RNA
PLCζ	phospholipase C zeta
PRM1	protamine 1
PRM2	protamine 2
PTM	post-translational modification
SCSA®	sperm chromatin structure assay
siRNA	small interference RNA
SIRT1	sirtuin 1, NAD^+-dependent deacetylase
sncRNA	small non-coding RNA
SUTI	sperm ubiquitin taq assay
TNP1	transition protein 1
TNP2	transition protein 2
TR-KIT	truncated form of the tyrosine-kinase receptor
UPS	ubiquitin-proteasome system
ϒH2AX	phosphorylation of histone H2AX

1 INTRODUCTION

1.1 Progenesis and implications for the fertility

Fertilization is defined as the fusion of oocyte and spermatozoa, giving rise to the embryo. Accordingly, the proper development of the gametes is a prerequisite for this (Kushnir *et al.*, 2017). The gamete's development begins from primordial germ cells (PGCs) during early embryo development and is accompanied by chromosome reduction to half and specific changes in epigenome to protect DNA and create a detailed plan for gene expression in subsequent offspring (Gosden, 2002; Rajender *et al.*, 2011; Sánchez and Smitz, 2012; De Felici, 2013; Griswold, 2016; Zheng *et al.*, 2016).

This time-involving development and differentiation of gametes is quite sensitive to external factors originating from the lifestyle of individuals. Both healthy or harmful habits have the potential to change epigenetic information in gametes and thus influence embryo fate (Schuh-Huerta *et al.*, 2012; Luderer, 2014; Estill and Krawetz, 2016; Buser *et al.*, 2018).

The first steps of oocytes and spermatozoa development are the same; both of them differentiate from pluripotent PGCs that are firstly detectable in the endodermal layer of the yolk sac on the 24th day after fertilization (Bukovsky *et al.*, 2005). Then they migrate to the genital ridge of the embryo, and their subsequent development into the oocytes or spermatozoa depends on the sex chromosomes in the somatic cell of the genital ridge (Morgan *et al.*, 2005; Coticchio *et al.*, 2013; De Felici, 2013).

1.2 Oogenesis

The oocyte development begins during prenatal development of the foetus, whereas PGCs are differentiated and anti-Müllerian hormone drives further development of female reproductive tract. This begins with creation of primordial follicles that are oogonia surrounded by granulosa cells (Findlay *et al.*, 2009; Adhikari and Liu, 2013; De Felici, 2013). While oogonia initiate their first meiotic division, granulosa cells divide mitotically and differentiate into cumulus cells (McNatty *et al.*, 2004; Sánchez and Smitz, 2012). The first round of meiosis is physiologically disrupted in diplotene when germinal vesicle (GV) is clearly defined. The first meiotic block is established in the prenatal stage of ontogenesis and is kept for several years until puberty onset in humans (Detlaf *et al.*, 1988; Demirtas *et al.*,

2010; Tripathi *et al.*, 2010). Thereafter, a part of the follicle pool is recruited for a growth, while the progress of the follicles and oocytes growth occurs. Meanwhile, oocyte growth is manifested by active proteosynthesis leading to a multiple increase in its volume (Eppig, 1992; Matzuk, 2002; Telfer and McLaughlin, 2011). Moreover, *zona pellucida*, the glycoprotein wrap of the oocyte, is synthesised. Re-initiation of meiosis is set in puberty with the first ovulation. This stage is called germinal vesicle breakdown (GVBD). Subsequently, the oocytes entry into the 1st Metaphase segregates chromosomes, the first polar body is extruded and the 2nd meiotic arrest is established (Detlaf *et al.*, 1988; Demirtas *et al.*, 2010; Tripathi *et al.*, 2010; Mehlmann, 2013). The meiotic maturation of oocyte, i.e. the process between the 1st and 2nd meiotic arrests, is highly unique and well-orchestrated by many kinases and factors of the ubiquitin-proteasome system (Mordret, 1993; Suzumori *et al.*, 2003; Huo *et al.*, 2004a, 2004b; Bodart, 2010; Nevoral *et al.*, 2014). Moreover, dynamic epigenetic changes, including many upstream regulators, are required, and many of them remain unelucidated (Fig. 1) (Dean, 2013; Luciano and Lodde, 2013; Zuccotti *et al.*, 2013; Adamkova *et al.*, 2017).

The matured oocyte is arrested in the 2nd meiotic block for a couple of hours, until the spermatozoon penetrates the *zona pellucida* and fuse with oolema. Thereafter, oocyte activation leads to completion of meiotic division and a 2nd polar body extrusion (Amdani *et al.*, 2015; Georgadaki *et al.*, 2016; Ohto *et al.*, 2016; Yeste *et al.*, 2017). The creation of male and female pronuclei occurs, followed by further embryonic development (Ge *et al.*, 2015; Chen *et al.*, 2019; Jansz and Torres-Padilla, 2019).

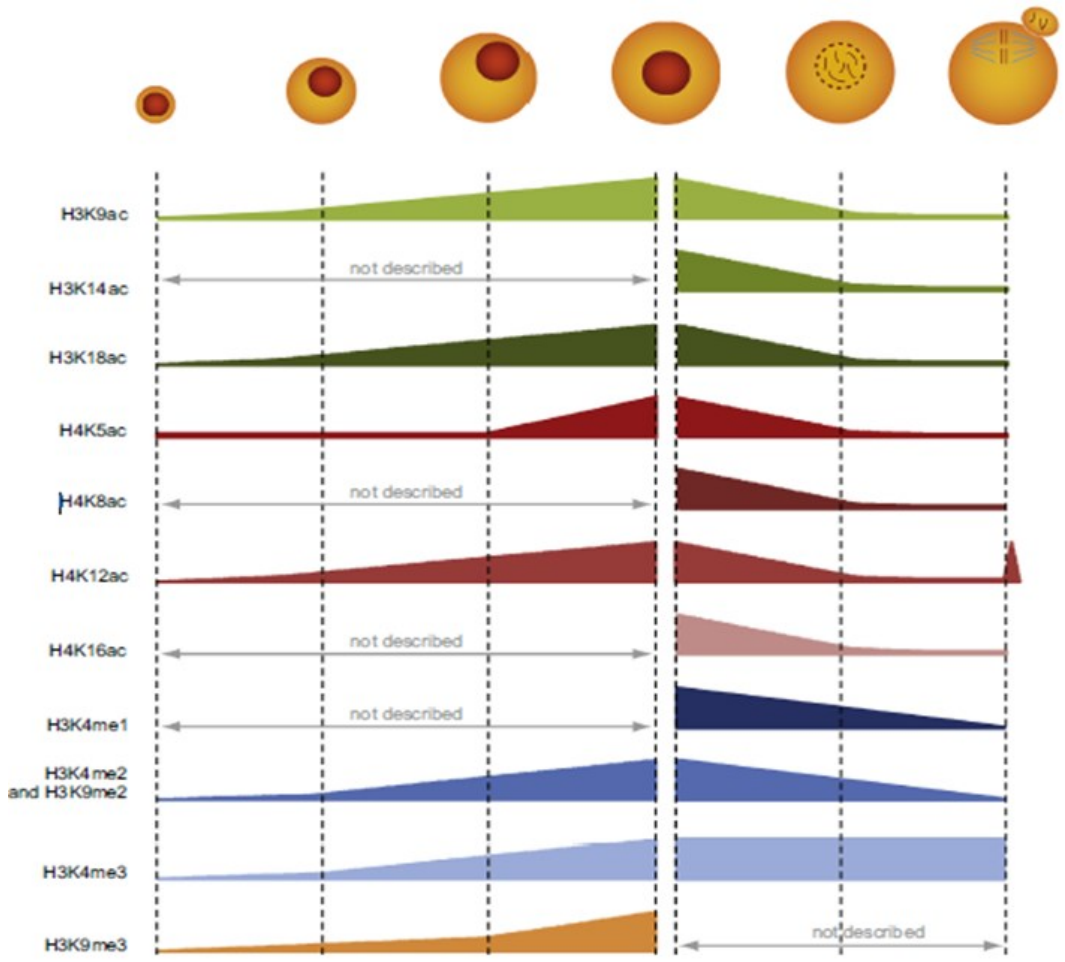

Figure 1. Overview of posttranslational modifications accompanying oogenesis (Clarke and Vieux, 2015).

1.3 Spermatogenesis

Spermatogenesis is the process of proliferation and differentiation of spermatogonial stem cells into adult spermatozoa within seminiferous tubules. In the first, diploid phase, spermatogonial stem cells multiply by mitosis and subsequently undergo meiotic division to reach the haploid phase, thus becoming spermatids (Holstein *et al.*, 2003; Cheng, 2008; Griswold, 2016). During meiotic division, in the pachytene stage, meiotic sex chromosome inactivation (MSCI) occurs. This mechanism is important in order to protect sex chromosomes against their reciprocal recombination during crossing over (Turner, 2007; de

Vries *et al.*, 2012; Kirkpatrick *et al.*, 2012). This is facilitated by histones posttranslational modifications and non-coding RNA (Hoyer-Fender, 2003; An *et al.*, 2010; Yao *et al.*, 2015). In the second phase, called spermiogenesis, spermatids begin their differentiation to spermatozoa. Formation of highly condensed chromatin and formation of acrosome and flagellum are typical for this process (Holstein *et al.*, 2003; Cheng, 2008; Griswold, 2016). Some of the steps of spermatozoa maturation are realized in epididymis (Sullivan and Mieusset, 2016).

Spermatogenesis encompasses distinct epigenetic events that are characterized as heritable changes in gene expression without changes in the nucleotide sequence, but with an influence on cell phenotype (Rajender *et al.*, 2011; Dada *et al.*, 2012; Donkin and Barrès, 2018). The epigenetic events of spermatogenesis include DNA imprinting and chromatin remodelling, leading to its condensation (Ferguson-Smith, 2001; Oliva, 2006; Castillo *et al.*, 2011; de Mateo *et al.*, 2011; Doshi *et al.*, 2013; Hiura *et al.*, 2014). These changes are defined by post-translational modifications (PTMs) of histone proteins (acetylation, methylation, phosphorylation, ubiquitination), small non-coding RNA and gradual replacement of histones by protamines, called protamination (Hazzouri *et al.*, 2000; Oliva, 2006; Baska *et al.*, 2008; Brykczynska *et al.*, 2010; Brunner *et al.*, 2014; Gannon *et al.*, 2014; Yuan *et al.*, 2016; Lambrot *et al.*, 2019; Schon *et al.*, 2019). Protamination provides to the spermatozoon a unique chromatin structure secured by toroidal structure created by protamines (Fig. 2) (Green *et al.*, 1994; Vilfan *et al.*, 2004; Balhorn, 2007). Its role is higher chromatin compaction and inactivation of spermatozoa genome to protect paternal DNA and conserve epigenetic information for an embryo (Carrell *et al.*, 2007; Oliva and Ballescà, 2012).

This process happens gradually during spermatid prolongation and is preceded by specific steps. The first step is chromatin relaxation caused by histone hyperacetylation, and subsequent attachment of bromodomain testis associated proteins (BRDT) as markers for the incorporation of transition protein 1 and 2 (TNP1, TNP2), which are fully replaced by protamines 1 and 2 (PRM1, PRM2) (Green *et al.*, 1994; Shang *et al.*, 2009; Gaucher *et al.*, 2012; Shirakata *et al.*, 2014). Their ratio in the mammalian sperm genome is usually set close to 1:1 and any shifts in this ratio have been related to male infertility (Wykes and Krawetz, 2003; Govin *et al.*, 2007). Protamines create specific disulphide bonds to prevent dislocation from DNA and similarly to histones, they are underlined by PTMs (Green *et al.*, 1994; Balhorn, 2007; Gaucher *et al.*, 2010). The best known of these is phosphorylation of serine,

threonine and tyrosine residues; however, their role has still not been described (Balhorn, 2007; Soler-Ventura *et al.*, 2018).

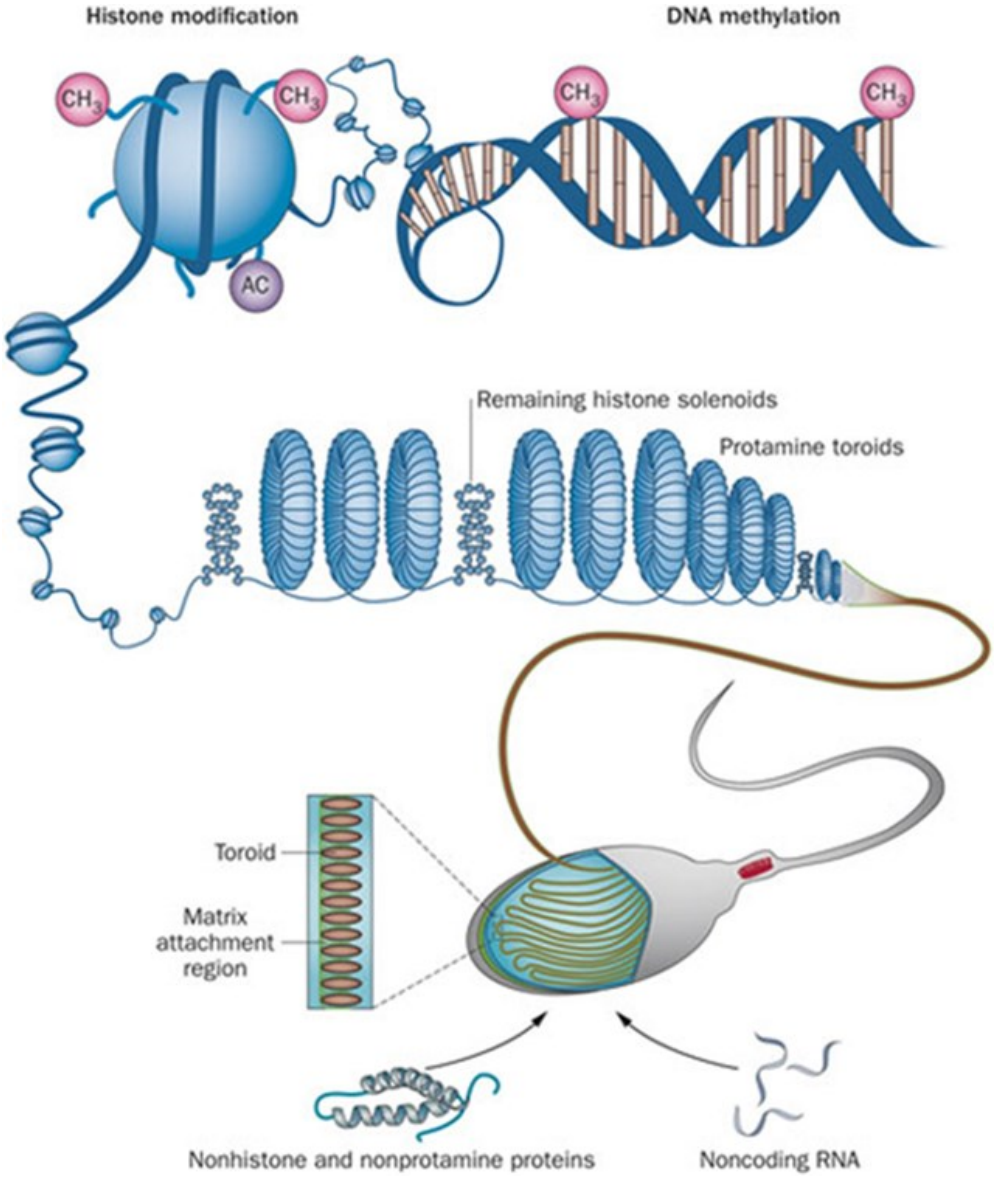

Figure 2. **Sperm chromatin** (Schagdarsurengin *et al.*, 2012). Spermatid elongation is typical for specific chromatin remodelling, called protamination. Its main purpose is the creation of a more-condensed chromatin structure to protect DNA against undesirable changes The process is accompanied by different epigenome based changes leading to chromatin relaxation and subsequent exchange of histones to protamines.

Moreover, chromatin structure, created by protamines, is apart from disulphide bonds keep by zinc bridges, that are specific binding between cysteine residues (Bjorndahl and Kvist, 2010). Zinc bridges are assumed to be created during maturation in epididymis and thus protect thiols against oxidation to disulphide bonds. Due to their reversibility, zinc bridges may be advantage for chromatin remodellation after fertilization (Bjorndahl and Kvist, 2010; Björndahl and Kvist, 2011, 2014). Apart from that zinc is part of zinc fingers domain of various proteins and transcriptional factors (Kerns *et al.*, 2018b). Hence, it is not surprising that amount of zinc in seminal plasma correlate with spermatozoa quality and recent study mapping four zinc signature across the capacitation as physiological event prior to fertilization, confirmed it (Kerns *et al.*, 2018a)

However, after protamination, a certain percentage of histones remains, the amount is species specific and varies from 1% in mice to 15% in human spermatozoa (Pittoggi *et al.*, 1999; Hammoud *et al.*, 2009; Balhorn *et al.*, 2018; Hamilton *et al.*, 2019). Residual histones undergo PTMs with the specific genomic position. Most residual histones are situated at the promoters of genes for spermatogenesis, housekeeping genes, non-coding RNAs (ncRNAs) and genes important for early embryo development (Brykczynska et al., 2010; Hammoud et al., 2011; Lambrot et al., 2019). Accordingly, spermatogenesis is responsible for the epigenetic setting of mature spermatozoa that are transcriptionally and translationally inactive (Fig. 3). Hence, spermatozoa completely rely on protein and small noncoding RNA source acquired during their development (Carrell and Hammoud, 2009; Brykczynska *et al.*, 2010; Siklenka *et al.*, 2015; Teperek *et al.*, 2016).

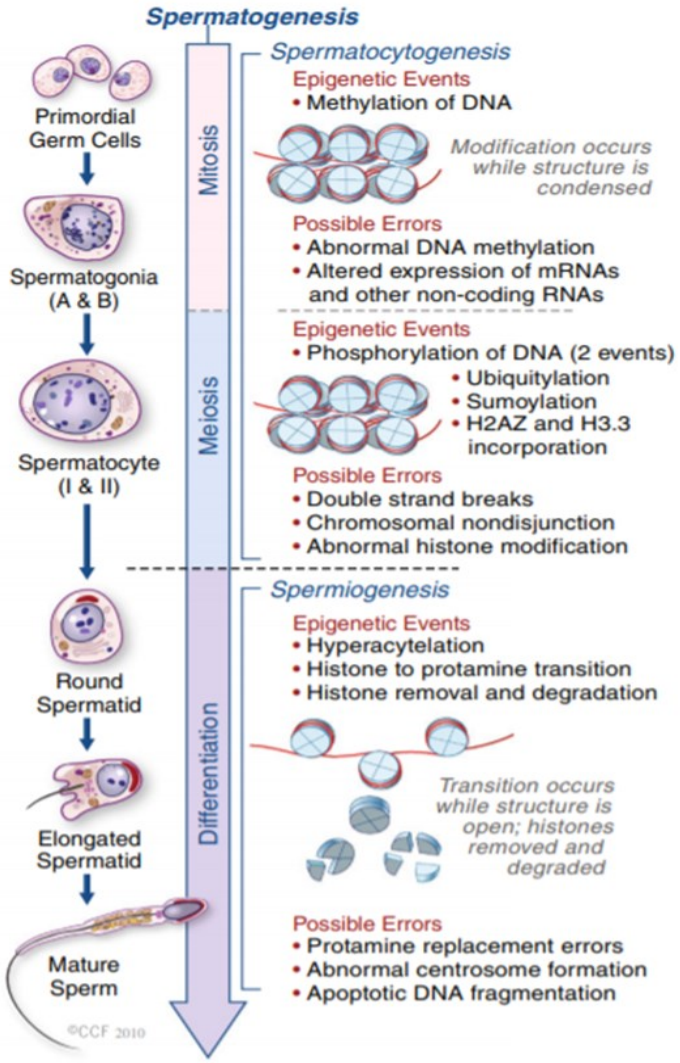

Figure 3. Review of the most important epigenetic events through spermatogenesis (Dada *et al.*, 2012). The diagram shows remodelling of chromatin, including posttranslational modifications across the stages of sperm development.

Currently, it is known that any disruption of histone to protamine replacement and abnormal PTMs of histones happens during spermatogenesis, and spermatozoa maturation could lead to male infertility (Speyer *et al.*, 2010; Stuppia *et al.*, 2015; Urdinguio *et al.*, 2015; Zhong *et al.*, 2015; Kutchy *et al.*, 2017; Tang *et al.*, 2018; Schon *et al.*, 2019). However, the sperm epigenome requires deep investigation for understanding its role and consequences for sperm fertilization ability and embryo development.

1.4 Sperm epigenome

1.4.1 Histone acetylation

Acetylation of protein is characterized by the transfer of an acetyl moiety from acetyl CoA to a free amino group of the target protein and in histones, it is catalysed by specific enzymes, namely histone acetyltransferases (HATs) and histone deacetylases (HDACs) (ALLFREY *et al.*, 1964; Sonnack *et al.*, 2002; Aly and Om, 2013). Histone acetylation is indispensable for chromatin remodelling, including histone removal by an ubiquitin-proteasome system (UPS) with subsequent replacement by protamines, as well as the regulation of gene expression (Hazzouri *et al.*, 2000; Haraguchi *et al.*, 2007; Rathke *et al.*, 2007; Paradowska *et al.*, 2012; Qian *et al.*, 2013).

Since acetylation is usually characterized as an activator of gene transcription and the ejaculated spermatozoa are transcriptionally inactive, histone acetylation plays a role mainly in spermatids to facilitate chromatin remodelling, leading to transcriptional inactivation in the sperm nuclei (Morinière *et al.*, 2009; Dhar *et al.*, 2012; Schon *et al.*, 2018). While acetylation of H4 is connected mainly to protamination, acetylation of H3 has a more regulatory function for spermatogenesis and embryo development (Dhar *et al.*, 2012; Kutchy *et al.*, 2017; Pipolo *et al.*, 2018). The reason for this could be that acetylation of histone H4 is recognized by BRDT proteins from the BET family, including BRDT1, BRDT2, BRDT3, and BRDT4, which represent the first step of histone to protamine replacement (Steilmann *et al.*, 2010; Dhar *et al.*, 2012; Gaucher *et al.*, 2012; Bryant *et al.*, 2015). BRDT is also characterized as a transcriptional factor that is highly expressed in round and elongating spermatids (Shang *et al.*, 2004). This group of protein participates in chromatin remodelling via two mechanisms, in the first step by recognition of hyperacetylated histone H4 and subsequently by binding to the Smarce1 protein, which is a member of the SWI/SNF family of ATP-dependent chromatin remodelling complexes (Dhar *et al.*, 2012; Gaucher *et al.*, 2012). Even though it seems that the role of BRDT proteins is just during chromatin remodelling, their presence in mature ejaculated spermatozoa point out their possible role during early embryo development. The mutation of the BRDT4 gene has a negative effect on an embryo and is associated with epilepsy and neural developmental defects. Altogether, BRDT-driven changes on the genomic or epigenomic level impact fertilization, embryo implantation, and development (Houzelstein *et al.*, 2002; Shang *et al.*, 2009, Shang *et al.*, 2011).

Histone hyperacetylation is also typical for spermatogonia that actively undergoes mitosis, and for round spermatids prior to elongation. In the round spermatids, increased acetylation of lysines K5, K8, K12 and K16 on H4 was detected. This state continues during spermatid elongation (Meistrich *et al.*, 1992; van der Heijden *et al.*, 2006; Morinière *et al.*, 2009). However, in mature spermatozoa, the amount of acetylated histones is lower and their position is functional for the regulation of gene expression in early post-fertilization stages of the embryo (Paradowska *et al.*, 2012; Luense *et al.*, 2016; Kutchy *et al.*, 2017). In the case of histone H3, there is evidence of acetylated lysines K9, K18 and K23, which are highly expressed in spermatogonia and in elongated spermatids what also indicate their role in protamination (Song *et al.*, 2011; Steilmann *et al.*, 2011; Kutchy *et al.*, 2017). Furthermore, while histone acetylation is related to chromatin relaxation, the level of HATs correlate with the increased DNA fragmentation and decrease sperm quality (Kim *et al.*, 2014).

Accordingly, any defect in histone acetylation constitutes a reason for improper chromatin compaction and is responsible for embryo failure (Carrell and Hammoud, 2009; Hammoud *et al.*, 2011; Simon *et al.*, 2011; Kim *et al.*, 2015; Kutchy *et al.*, 2017; Oumaima *et al.*, 2018). This is also proven by the study of Berkovits and Wolgemuth (2011), who found out that BRDT domain keeps sirtuin 1 (SIRT1, a NAD^+-dependent histone deacetylase) out of pericentric chromatin, which is supposed to be intact during protamination. Ablation of SIRT1 is responsible for abnormal histones-protamine replacement and defects of chromatin condensation in spermatids. Moreover, knockout of *Sirt1* is related to aberrant spermatogenesis and decrease fertility (Coussens *et al.*, 2008; Bell *et al.*, 2014).

1.4.2 Histone and DNA methylation

Histone acetylation is counterbalanced by methylation events that usually have an opposite effect on chromatin condensation and gene expression. However, there are also some exceptions (Hammoud *et al.*, 2011; Siklenka *et al.*, 2015; Lambrot *et al.*, 2019; Schon *et al.*, 2019). The most studied of these have been the mono-, di- and trimethylation of H3 and H4, which were detected during certain stages of spermatogenesis and in ejaculated spermatozoa (Krejčí *et al.*, 2015; Luense *et al.*, 2016).

While in spermatogonia and in elongated spermatids, most of the histones are demethylated to allow for gene expression of proteins important for spermatogenesis

chromatin remodelling (Waterborg, 2002; Aoki *et al.*, 2005; Rathke *et al.*, 2014). In ejaculated spermatozoa, the residual histones are usually methylated to preserve genetic information by preventing undesirable changes and possible DNA fragmentation for the embryo (Gannon *et al.*, 2014; Luense *et al.*, 2016; Jenkins *et al.*, 2017). Hitherto, this is known about methylation of lysine K4, K9, K27, K36 and K79 of histone H3 and lysine K20 of histone H4 (Hammoud *et al.*, 2009). Most histone methylations are associated with heterochromatin establishment (Sims *et al.*, 2003); however H3K36, H3K79, and H3K4 surprisingly occur in euchromatin (Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010; Lambrot *et al.*, 2019; Schon *et al.*, 2019). In particular H3K4me1/2/3 is intensively studied and many molecular implications in spermatogenesis, fertilization and early embryonic development are presumed (Godmann *et al.*, 2007; Rathke *et al.*, 2007; Brykczynska *et al.*, 2010; Hammoud *et al.*, 2011).

The important role of methylated histone for spermatogenesis and embryo development is also proven by the changes in their level through various stages of sperm development. While mono-, di-, tri-methylation of H3K4 increase in pre-leptotene spermatocytes, it then decreases to be overexpressed again in spermatids prior to chromatin remodelling (Godmann *et al.*, 2007; Rathke *et al.*, 2007; Teperek *et al.*, 2016). Upregulation of histone methylation justifies that histone hyperacetylation is not only one modification important for histones to protamine exchange, but rather, a complex coordination of all histone PTMs play a crucial role for the creation of properly condensed mature sperm chromatin. Moreover, the position of some methylated histones was elucidated and helps to uncover their function. H3K4me2 used to be situated at the promotors of housekeeping genes and genes coding small ncRNAs (Brykczynska *et al.*, 2010; Wilson *et al.*, 2018).

Similarly to H3K4me2, tri-methylation of H3K27 was associated with the genes responsible for embryo development (Brykczynska *et al.*, 2010). However, contrary to H3K4me2, H3K27me3 marks genes that are silenced during early embryo development (Hammoud *et al.*, 2009). The specific group of genes are HOX, which are evolutionally conserved and marked bivalently with H3K4me2 and H3K27me3 (Carrell and Hammoud, 2009; Brykczynska *et al.*, 2010).

Histone methylation is regulated by methyltransferases (HMT) and demethylases, whose level also varies during spermatogenesis, and their expression is an indicator of methylation status in the sperm chromatin (Teperek *et al.*, 2016; Eelaminejad *et al.*, 2018).

For example, JmjC domain-containing histone demethylase 2A (JHDM2A; also known as JMJD1A and KDM3A) binds to promoters of genes for TNP1 and protamine PRM1 to demethylate H3K9 and, in this way, allow TNPs and protamines expression and enable chromatin remodelling (Okada *et al.*, 2007, Okada *et al.* 2010; Liu *et al.*, 2010; Eelaminejad *et al.*, 2017). Accordingly, the level of histone demethylase is related to male fertility. It was proven that azoospermic males with downregulation of JHDM2A have lower expression of transition protein and protamines. This study confirms the importance of JHDM2A for spermatogenesis (Okada *et al.*, 2007; Javadirad *et al.*, 2016). On the contrary, H3K9me is methylated by SUV39H1, which preserves genomic stability by protection pericentric chromatin, which is also created by H3K9me (Peters *et al.*, 2001).

Another histone demethylase is lysine-specific histone demethylase (LSD1/KDM1A), whose target is H3K4me2. Ablation of LSD1 in the testes causes accumulation of apoptotic germ cells and disruption of spermatogenesis (Lambrot *et al.*, 2015; Siklenka *et al.*, 2015; Myrick *et al.*, 2017). Similarly, Siklenka *et al.* (2015) observed that overexpression of LSD1 during spermatogenesis cause the loss of H3K4me2 from promotors of genes important for development; furthermore alternation of the RNA profile in sperm and offspring was detected. These changes were transmitted through three generations and phenotypically cause lower offspring's surveillance.

Accompanying histone methylation, DNA methylation takes place in the genome, representing an important tool for adequate sperm quality achievement (Stuppia *et al.*, 2015; Sujit *et al.*, 2018). DNA methylation is defined as the binding of the methyl unit from the S-adenosyl-L-methionine to the 5^{th} position of cytosine residues in nucleotides using DNA methyltransferases (DNMTs). DNA methylation is another controlling mechanism in the sperm chromatin. DNA methylation usually happens during cytosine-phosphate-guanine (CpG) dinucleotides and is regulated by DNMTs, which are responsible for methylation maintenance and *de novo* methylation (Okano *et al.*, 1999; Jones, 2012).

1.4.3 Histone phosphorylation

Histone phosphorylation is a quite different modification than acetylation or methylation. The reason for this is that it doesn't directly influence gene expression in the way of activation or repression (Fernandez-Capetillo *et al.*, 2003; Vigodner, 2009; de Vries *et*

al., 2012; Zhong *et al.*, 2015). The role of histone phosphorylation is mainly the transformation of the nucleosomes surface and recruitment of other protein complexes for chromatin remodulation. Phosphorylation occurs by adding the phosphoryl moiety to the serine residues of histones. The most know phosphorylated histones are H1, H2A, and H2B, and they are joined to different processes like meiotic sex chromosome inactivation (MSCI), protamination, DNA reparation, and others. However, the most studied phosphorylated histone is H2A (Fernandez-Capetillo *et al.*, 2003; Li *et al.*, 2006; de Vries *et al.*, 2012; Garolla *et al.*, 2015; Zhong *et al.*, 2015; Tsuribe *et al.*, 2016).

Phosphorylated H2A (ϒH2AX) is one of the best establish hallmarks of DNA fragmentation, which is relevant not just for spermatozoa, but also for many other cell types (Li *et al.*, 2006; Shamsi *et al.*, 2008; Wang *et al.*, 2019). It was proven that at the site of DNA fragmentation, the H2AX histone variant is recruited and subsequently phosphorylated by the specific protein group phosphatidylinositol 3-kinase-related kinases (PIKKs), for example, ataxia-telangiectasia mutated (ATM), ataxia- and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) (Blanco-Rodríguez and Blanco-Rodriguez, 2012). Mobilization of these proteins at the site of DNA damage is the first step of DNA reparation. Subsequently, a mediator of DNA damage checkpoint 1 (MDC1) is recruited and phosphorylated by ATM to stabilize DNA damage factors, for example, 53BP1, BRCA1, Rad51, NBS1 and TopoBP1 that were found to be colocalized with the ϒH2AX. All of them were confirmed as indispensable for DNA reparation through genomic protection and cell cycle regulation. Subsequently, after DNA reparation, ϒH2AX is dephosphorylated (Celeste *et al.*, 2002; Li *et al.*, 2006; Vasileva *et al.*, 2013; Ahmed *et al.*, 2015; Mayer *et al.*, 2016). Li *et al.* (2006) showed that oxidative stress causing DNA fragmentation in sperm recruits ϒH2AX and is accordingly a promising marker of DNA damage. Moreover, the level of ϒH2AX was significantly higher in infertile men and negatively correlates with sperm quality evaluated by concentration, motility, and morphology (Zhong *et al.*, 2015). Accordingly, a negative effect on embryo cleavage, embryo quality, and pregnancy rates were observed (Tsuribe *et al.*, 2016).

This mechanism of DNA reparation involving ϒH2AX is crucial not just for damage caused by the surrounding environment, but DNA breaks happen during spermatogenesis physiologically (Marcon and Boissonneault, 2004; Laberge and Boissonneault, 2005; Leduc *et al.*, 2008). ϒH2AX plays the same role during physiological breaks, for example during

spermatogenesis, prior to protamination (Fernandez-Capetillo *et al.*, 2003). The meiosis recombination when the genetic information between homologous chromosomes is exchanged is typical or meiosis. To secure chromosome integrity and thus correct oocyte maturation, ϒH2AX is involved in the response to DNA breaks and their subsequent reparation (Mayer *et al.*, 2016). Furthermore, ϒH2AX is also important for MSCI to prevent exchanging of genes between X and Y chromosomes; the sex bodies have to be covered by ϒH2AX (Fernandez-Capetillo *et al.*, 2003; de Vries *et al.*, 2012).

Altogether, ϒH2AX is histone PTM whose role is mainly DNA protection and prevention of tumorigenesis and DNA reparation - all of these processes are important during all cell life. Since spermatozoa are sensitive to the oxidative stress, the main cause of DNA damage, the DNA reparation process initiated by ϒH2AX is crucial to protect genetic information for the embryo and as prognostic sperm quality marker (Zhong *et al.*, 2015; Tsuribe *et al.*, 2016; Wang *et al.*, 2019).

1.4.4 Histone ubiquitination and ubiquitin-proteasome system

Ubiquitination is ATP-dependent PTMs happening at lysine residue by attachment of small chaperone molecule, containing only 76 amino acid, called ubiquitin. This process is performed by three enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin conjugating enzyme (E2), and the ubiquitin ligase enzyme (E3). The third enzyme used to be tissue-specific and accordingly to that, has different names (Glickman and Ciechanover, 2002; Hou and Yang, 2013; Przedborski *et al.*, 2016).

Ubiquitination is involved in spermatogenesis and epididymal maturation as a quality control mechanism to eliminate defective spermatozoa and dead epididymal epithelial cells (Baska *et al.*, 2008; Sheng *et al.*, 2014). During spermatogenesis, ubiquitin participates in the degradation of different proteins or organelles via the ubiquitin-proteasome system; for example, histones during chromatin remodelling (Bose *et al.*, 2014; Sheng *et al.*, 2014; Dong *et al.*, 2016). Histones with destiny to be removed are marked by the chain of ubiquitin molecules and subsequently eliminated by the 26S proteasome, recognizing ubiquitin chains (Singh *et al.*, 2009; Bach and Hegde, 2016). Furthermore, ubiquitin, secreted from principal epididymal epithelial cells, participates in the degradation of defective spermatozoa that had been marked by ubiquitin molecules. Thereafter, defective spermatozoa are eliminated by

phagocytosis (Sutovsky *et al.*, 2001; Baska *et al.*, 2008; Richburg *et al.*, 2014). Studies have shown that ubiquitinated spermatozoa have damaged DNA, aberrant chromatin condensation, signs of early apoptosis and point out ubiquitin as an important quality controller for spermatozoa passing epididymis (Ozanon *et al.*, 2005; Baska *et al.*, 2008; Hosseinpour *et al.*, 2014; Sutovsky *et al.*, 2015).

Other targets of ubiquitination are histones, regulating gene expression. In male reproduction, the most known ubiquitinated histones are H2A (H2Aub), H2B (H2Bub) and H2AX (H2AXub), H2AK119 (H2AK119ub) (An *et al.*, 2010; Ma *et al.*, 2011; Xu *et al.*, 2016; Nevoral *et al.*, 2019 in press). Their function is similar to phosphorylated ϒH2AX, DNA reparation, MSCI, and protamination (An *et al.*, 2010; Sheng *et al.*, 2014). At the DNA damage sites, RNF8 E3 ligase is recruited by the mediator of DNA damage checkpoint 1 (MDC1) and, H2A, H2B and H2AX are ubiquitinated directly there. Subsequently, the other above-mentioned factors important for DNA reparation are mobilized (Jiang *et al.*, 2018). The lack of RNF8 in the testes causes a decreased level of histone acetyltransferase MOF, which effect defect in chromatin relaxation and decrease the amount of transition proteins and protamines (Ma *et al.*, 2011). This finding points out the dependency of histone ubiquitination to chromatin relaxation caused by histone hyperacetylation (Ma *et al.*, 2011). In addition to histone ubiquitination as a DNA damage-mark, H2AK119 is also known as a heterochromatin repressive mark (Nevoral *et al.*, 2019 in press). This study points out the ambiguous character of histone ubiquitination that must be observed.

Tightly related to the ubiquitination is SUMOylation, which is characterized by the attachment of a small ubiquitin-like modifier (SUMO) to a lysine residue of certain protein (Geiss-Friedlander and Melchior, 2007). There are currently four known SUMO isoforms: SUMO1, SUMO2, SUMO3, and SUMO4. Their function is mainly in chromatin inactivation and repression of gene expression (Gill, 2004; Jürgen Dohmen, 2004; Geiss-Friedlander and Melchior, 2007; Brown *et al.*, 2008). Whereas SUMO1 was colocalized with proteins HP1, H3K9me3 and H4K20me2, it seems that sumoylated histones play role in the maintenance of constitutive chromatin and the localization of SUMO1 on the sex chromosome during the first round of meiosis, indicating its role in MSCI (Vigodner, 2009; Stielow *et al.*, 2010; Maison *et al.*, 2016). Moreover, dynamin-related protein 1 (DRP1), Ran GTPase-activating protein 1 (RanGAP1) and Topoisomerase IIa are sumoylated in the somatic cells and germ cells; however, morphologically abnormal spermatozoa and spermatozoa of asthenozoospermic

males have significantly increased the level of SUMOylation and, specifically, SUMOylation of these proteins was also higher (Marchiani *et al.*, 2014). Based on this, SUMOylation could be other sperm quality control with possible application to assisted reproductive therapy (ART) (Marchiani *et al.,* 2011; Vigodner *et al.*, 2013; Marchiani *et al.,* 2014).

1.4.5 Other post-translational modifications of histones

The newest histone PTMs in sperm are crotonylation and butyrylation. Both modifications seem to be a hallmark of active transcription (Montellier *et al.*, 2012; Goudarzi *et al.*, 2016).

Crotonylation (Krc) is created by binding the crotonyl group to a lysine residue and is situated mainly at the promotors of sex-linked genes. All core histones were identified as the targets for crotonylation. A strong Krc signal was observed in the elongated spermatids. Nevertheless, the level of Krc did not correlate with higher gene expression in the meiotic and postmeiotic cells, it was observed that most of the genes marked by Krc in round spermatids were expressed in postmeiotic stages. The localization of Krc was observed at the post-meiotically activated genes on autosomes promotors or sex chromosomes. This finding confirms the role of Krc for gene expression of haploid cells. Moreover, the distribution of crotonylated histones in the round and elongated spermatids also indicates their role during protamination and MSCI (Tan *et al.*, 2011; Montellier *et al.*, 2012; Liu *et al.*, 2017b).

Histone butyrylation also occurs in lysine residues; however, contrary to other histones PTMs, it is not situated just at the N-terminal tail domains, but also in other regions of core histones, which could explain its coexistence with other PTMs. The butyrylation was identified at the histone H4, H3, and H2B. The histone modification was observed in spermatogonia, spermatocytes, and spermatids and subsequently butyrylated histones were removed from chromatin. Interestingly, it was proven that butyrylated histones stay in the chromatin longer than acetylated histones, the reason for this could be that butyrylation slows down histone to protamine replacement and inhibits Brdt binding. This altogether indicates the specific role of butyrylation during protamination (Chen *et al.*, 2007; Goudarzi *et al.*, 2016).

There exist many other histone PTMs, such as propionylation, malonylation, succinylation, serotonylation, and GlcNAcylation. Most of them were found using mass

spectrometry (Lewis and Hanover, 2014; Kebede *et al.*, 2017; Yokoyama *et al.*, 2017; Ishiguro *et al.*, 2018; Farrelly *et al.*, 2019). However, their role in spermatozoa or during spermatogenesis is still not clear.

1.4.6 Non-coding RNAs

The definition of ncRNAs comes from their name. They are small molecules of RNA that are not translated to proteins and are involved in the regulation of gene expression, especially gene silencing in gametes and the embryo (Amanai *et al.*, 2006; Schuster *et al.*, 2016; Svoboda, 2017). There exist many types of ncRNAs, but they can be classified as small non-coding RNAs (snRNAs) and long non-coding RNAs (lncRNAs). Small interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs) belong to the group of snRNA (Bernstein *et al.*, 2001; Lee *et al.*, 2003; Girard *et al.*, 2006).

MicroRNA (miRNA) is small RNA with the length approximately 20 nucleotides, creating a functional complex with AGO proteins (a subfamily of Argonaute protein family), called miRNA-induced silencing complex (miRISC) (Mciver *et al.*, 2012). The origin of miRNA comes from genes for miRNA that are transcribed to long primary RNA (priRNA) by RNA polymerase II (pol II), which is cut by double-stranded RNA endonuclease DROSHA to make shorter priRNA in the nucleus and subsequently transported from the nucleus through transporter protein exportin 5 into the cytoplasm (Kim *et al.*, 2009; Inui *et al.*, 2010; Krol *et al.*, 2010)(Kim *et al.*, 2009; Winter *et al.*, 2009; Krol *et al.*, 2010). There, priRNA is again cleaved by endoribonuclease DICER that generate mature miRNA, creating miRISC with AGO proteins. The role of miRISC is in degradation or silencing mRNAs (Winter *et al.*, 2009). It was proven that miRNA have a regulatory function during spermatogenesis (He *et al.*, 2009; Mciver *et al.*, 2012; Kotaja, 2014; Salas-Huetos *et al.*, 2014). miR-34c is probably the most studied miRNA, highly expressed in spermatogonia and spermatids with the key role in the embryo cleavage. However it is not present in oocyte (Bouhallier *et al.*, 2010; Liu *et al.*, 2012; Cui *et al.*, 2015). Currently, hundreds of miRNAs have been identified; many of them are spermatogonia, spermatocytes, spermatid or spermatozoa specific, highlighting their role in all phases of sperm development. In connection with male fertility, differences in miRNA expression between asthenozoospermic, oligoasthenozoospermic, azoospermic samples

compared to the control group were detected (Krawetz *et al.*, 2011; Kawano *et al.*, 2012; Salas-Huetos *et al.*, 2014; Yuan *et al.*, 2016; Barceló *et al.*, 2018; Li *et al.*, 2018).

Small interference RNA (siRNA) is double-stranded RNA of 21 nucleotides that have similar genesis to miRNA. siRNA found its application in knockdown of specific genes what seems to be better for studying gene function that complete gene knockout, usually leading to lethality (Tolia and Joshua-Tor, 2007; He *et al.*, 2009; Krawetz *et al.*, 2011).

Piwi RNA (piRNA) is single-stranded RNA, approximately 24-30 nucleotides long (Siomi *et al.*, 2011). The piRNA is Dicer independent and, compared to miRNA that is expressed in all tissues and cells, piRNA is specifically expressed in the testes and its expression in spermatocytes and spermatids was also observed. piRNA got their name according to the second subfamily of the Argonaute protein family, called PIWI, in addition to MIWI, MIWI2 and MILI that are expressed differently across the spermatogenesis stages (Klattenhoff and Theurkauf, 2008; Zhang *et al.*, 2015b). While MILI and MIWI2 are already expressed in prospermatogonia in the foetus, the expression of MIWI starts after birth and mainly in pachytene spermatocytes and round and elongating spermatids (Zhang *et al.*, 2015b). Mutation in MILI and MIWI2 leaded to removing of DNA methylation at the transposomes, what was manifested by infertility. Furthermore, there the ability of piRNA to reproduce itself and silence transposons during de novo methylation was observed. Accordingly, the main role of piRNA is transposomes silencing and the regulation of de novo methylation (Kuramochi-Miyagawa *et al.*, 2008).

Long non-coding RNA (lncRNA) has a length of more than 200 nucleotides and could serve as a source for DROSHA and DICER enzymes to produce snRNAs (Mercer *et al.*, 2009; Zhang *et al.*, 2016; Svoboda, 2017). However, another function of lncRNA is silencing through interaction with proteins or direct binding to DNA (Kurihara *et al.*, 2014; Luk *et al.*, 2014; Zhang *et al.*, 2016; Svoboda, 2017). Apparently, the most know lncRNA is coding by imprinted gene H19 that is methylated in paternal line and expressed from the maternal allele (Keniry *et al.*, 2012).

Whereas spermatozoa are transcriptionally inactive, ncRNAs play an indispensable role for their proper function (Itou *et al.*, 2015; Holt *et al.*, 2016; Sharma *et al.*, 2018). Ablation of Dicer protein responsible for miRNA and siRNA production has a serious effect on sperm development and maturation, evident from a lower amount of germ cells, disruption of chromatin remodelling and spermatid elongation and infertility (Kim *et al.*, 2010; Romero *et*

al., 2011). Moreover, fertile and infertile individuals differ in ncRNAs expression profile (Abu-Halima *et al.*, 2014; Zhang *et al.*, 2015a; Salas-Huetos *et al.*, 2016). Interestingly, there are studies describing that during maturation in the epididymis, spermatozoa obtain a package of ncRNAs from small extracellular vesicles involved in the epididymis (Belleannée, 2015; Holt *et al.*, 2016; Sharma *et al.*, 2018). This package of ncRNAs is involved in sperm function, embryo development, and transgenerational epigenetic memory (Rodgers *et al.*, 2015). The necessity of sperm-borne RNAs for embryo was proven by ICSI when using spermatozoa of knockout DROSHA. DICER knockout mice have a negative effect on the embryo development potential; that was rescued after subsequent microinjection of total ncRNAs or snRNAs (Yuan *et al.*, 2016). Based on all known information, ncRNAs look like another candidate marker with diagnostic potential for ART.

1.4.7 Epigenetic double erasure and gene imprinting

Genetic imprinting is epigenetic regulation of gene expression when paternal or maternal allele is expressed in the embryo (Li and Sasaki, 2011). This parent-of-origin gene manifestation is ensured mainly due to DNA methylation and the on/off switching of the gene from the maternal or paternal line (Lin *et al.*, 2003; Ferguson-Smith, 2011). Imprinting genes used to be situated at imprinting control regions (ICRs), rich at CpG sites, undergoing DNA methylation. Another name for these regions is differentially methylated regions (DMRs) (Feil, 2009; Shin *et al.*, 2011). However, these regions are also regulated by residual histone PTMs and small non-coding RNA (snRNA) (Wilkins, 2006; Watanabe *et al.*, 2011; Guseva *et al.*, 2012; Jones, 2012).

Imprinted genes are responsible for embryo development, placental formation, brain function, and metabolism (Smith *et al.*, 2006; Wilkinson *et al.*, 2007). Accordingly, any aberration in the genes or their regulation has a detrimental effect on the embryo, usually manifested by certain congenital disorders (Hirasawa and Feil, 2010). Currently, there are known more than 100 imprinted genes, and most of them are methylated in the maternal line (Monk *et al.*, 2019). However, there three paternally methylated ICRs, *H19/Igf2*, *Gtl2/Dlk1* and *Rasgrf* are well described (Yoon *et al.*, 2002; Lin *et al.*, 2003; Doshi *et al.*, 2013).

The ICR region *H19/Igf2* at chromosome 7, involves a CTCF-dependent insulator that enables expression of the paternal allele for insulin-like growth factor 2 (*Igf2*), and repression

of *H19* (Bell and Felsenfeld, 2000; Doshi *et al.*, 2013). *Igf2* is the gene for the receptor of the growth hormone insulin-like growth factor type 2 and *H19* for long non-coding RNA (Leighton *et al.*, 1995). The *H19* is silenced by few mechanisms, DNA methylation, histones PTMs and ncRNA. There was proven enrichment of H3K4me3 at maternal allele for *H19*, and H3K9me3 at paternal allele for *H19* (Lee *et al.*, 2010; Doshi *et al.*, 2013). To contrary maternal allele for *Igf2* is repressed by ncRNA *Airn* (Sleutels *et al.*, 2002).

Gene imprinting is associated with the cycle of 'erasure' and 're-writing' of the imprinting in the pattern of parent-of-origin (Sun *et al.*, 2017). Firstly, the epigenetic code is erased, while terminally differentiated gametes are fused and dedifferentiated zygote developed (Oswald *et al.*, 2000; Smith *et al.*, 2012). This erasure leads to totipotent zygote creation, allowing cell differentiation through embryonic development (Oswald *et al.*, 2000; Boyano *et al.*, 2008; Hackett *et al.*, 2013; Zheng *et al.*, 2016). The first erasure does not affect imprinted genes and other erasure-resistant loci. However, even gene imprinting is erased in differentiated PGCs and, therefore, does not affect the whole embryo (Mitsuya *et al.*, 1998; Hajkova *et al.*, 2002). Adequate gene imprinting occurs in PGCs as well as X-chromosome is inactivated whereas PGC differentiation is running (McCarrey and Dilworth, 1992; Eggan *et al.*, 2000; Hajkova *et al.*, 2002; Li *et al.*, 2004; Keniry and Blewitt, 2018)..

There are many consequences of errors in gene imprinting, such as Beckwith–Wiedemann syndrome (paternally expresses *H19*), Angelman, Prader-Willi, and Silver-Russell syndromes (Hirasawa and Feil, 2010). The incidence of these disorders is increasing with the using ART, specifically intracytoplasmatic sperm injection (ICSI), and pointed out bypassing natural selections barriers involving sperm epigenetic (Chopra *et al.*, 2010; Tang *et al.*, 2017; Lou *et al.*, 2018; Hattori *et al.*, 2019). Moreover, an association of imprinting disorders and ART embryo culturing was found, which highlights the importance of epigenetic in gametes (Hirasawa and Feil, 2010; Anckaert *et al.*, 2013), and its study to improve ART and avoid the risk of imprinted disorders and others caused by a defect in epigenetic mechanisms.

1.5 Gasotransmitters as a new modulator of histone code

Gasotransmitters are defined as endogenously-produced small gaseous molecules with signalling function in various cells and tissues, and their production is secured by specific

enzymes. Although they are membrane permeable, they can easily get near the target. Three molecules belong to the gasotramitters family, namely nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H_2S) (Palmer *et al.*, 1987; Wang, 2002; Wu *et al.*, 2018). Their role is most investigated in the nervous and vascular system, where they behave as second messenger molecules as well as posttranslational modifiers of proteins (Vandiver *et al.*, 2013; Zhang and Bian, 2014; Kimura, 2015; Sen, 2017; Koriyama and Furukawa, 2018; Martelli *et al.*, 2019).

The first known gasotransmitter molecule is nitric oxide, which is produced by three isoforms of nitric oxide synthase (NOS) in all cells (Palmer *et al.*, 1987). Accordingly, it is the most investigated gasotransmitter in reproduction (Thaler and Epel, 2003; Lee and Cheng, 2008; Vignini *et al.*, 2008; Lefièvre *et al.*, 2009; Basini and Grasselli, 2015; Jovicić *et al.*, 2018). NO participates in folliculogenesis and oocytes maturation (Thaler and Epel, 2003; Pandey *et al.*, 2010; Basini and Grasselli, 2015). Moreover, NO participates in the regulation of cyclic changes in the uterus by modulation of smooth muscle contractility (Bao *et al.*, n.d.; Hertelendy and Zakar, 2004). The production of NO by oviduct and cumulus cells of oocytes has an important signalling function for spermatozoa heading to fertilize oocytes (Rosselli *et al.*, 1996; Ekerhovd *et al.*, 1999; Hattori *et al.*, 2001; Reyes *et al.*, 2004). It was found that NO is an important signalling molecule promoting their capacitation (Miraglia *et al.*, 2007; Machado-Oliveira *et al.*, 2008). The possible modus operandi is through the regulation of tyrosine phosphorylation and acrosomal reaction (Herrero *et al.*, 1997, 1999; Aitken *et al.*, 2004; de Lamirande and Lamothe, 2009). Lefievre et al (2009) and Machado-Oliveira *et al.* (2008) showed that different proteins involved in capacitation undergo nitrosylation in human spermatozoa, which may be another way in which gasotransmitters are involved in the regulation of different physiological events of spermatozoa (Coussens *et al.*, 2008; Bell *et al.*, 2014).

Similarly, the indispensable role of H_2S was proven in female reproduction (Nevoral *et al.*, 2016). H_2S is produced endogenously by three H_2S-releasing enzymes involved in cysteine metabolism, Cystahionine β-synthase (CBS), Cystathionine ϒ-lyase (CTH) and 3-Mercaptopyruvate sulfurtransferase (MPST) (Mustafa *et al.*, 2009). All three enzymes were detected in oocytes (Nevoral *et al.*, 2015). Furthermore, H_2S participates in folliculogenesis and oocytes maturation through interaction with maturation/M-phase promoting factor (MPF) and mitogen activated protein kinase (MAPK) (Nevoral *et al.*, 2014). Apart from this, the

protective anti-aging effect of H_2S for oocytes was also observed (Krejcova *et al.*, 2015; Nevoral *et al.*, 2018b).

Recently, the role of H_2S was investigated in sperm physiology (Wang *et al.*, n.d.; Li *et al.*, 2015b). All the three H_2S releasing enzymes, CBS and CTH were detected in the testes and germ cells (Sugiura *et al.*, 2005). Wang et al (2018) have observed lower levels of H_2S in seminal plasma of asthenospermic male, and artificial supplementation of H_2S was able to reverse hyperactivated movement of spermatozoa. Moreover, CBS knockout mice have lower sperm concentration and motility compared to wild mice (Wang *et al.*, 2018). In accordance with this, Li *et al.* (2015) investigated the protective effect of H2S against oxidative stress caused by heat stress during spermatogenesis. On the other hand, last year a study detected sulfhydration of histone NAD^+-dependent histone deacetylase SIRT1 in aorta and liver cells. This post-translational modification increases SIRT1 deacetylase activity and thus protects mice against atherosclerosis (Du *et al.*, 2018). However, SIRT1 play an indispensable role in sperm protamination, and thus its regulation by gasotransmitters is possible.

These findings showed how little we still know about molecular processes in the cells. Furthermore, it offers a new view of epigenetics, and hence possible regulation of histone posttranslational modifications by gasotransmitters. Nevertheless, these molecules are quite new in male reproduction and their role is undeniable. However, understanding of their involvement in sperm physiology is missing, and it is thus necessary to investigate them; this may also completely change the view of sperm function and epigenetic.

1.6 Paternal contribution in embryo development

For many years, spermatozoa were exclusively considered as a transporter of paternal DNA; however, this is no longer true, and many studies investigated paternal contributions in the fertilization process and embryo development (Sutovsky and Schatten, 2000; Jenkins and Carrell, 2011). In addition to DNA, sperm participates in fertilization by sperm-borne oocyte activating factors (SOAFs), centriole, mRNAs, and ncRNAs (Sutovsky and Schatten, 2000; Boerke *et al.*, 2007; Yeste *et al.*, 2017). However, recently many sperm-borne factors carrying specific instructions for the fertilization and embryonic development have been identified.

The fertilization process is successfully completed when oocyte is activated to finish second meiosis, maternal and paternal pronuclei is formed, which is to be subsequently

merged and create a zygote (Georgadaki *et al.*, 2016). For release of an arrested oocyte from the second metaphase, SOAF is crucial. Phospholipase C zeta (PLCξ), post-acrosomal WW-domain binding protein (PAWP), and a truncated form of the tyrosine-kinase receptor (TR-KIT) belong to this family (Tavalaee and Nasr-Esfahani, 2016).

PAWP is part of the sperm post-acrosomal sheath of the perinuclear theca. Its final position is obtained during spermatid elongation when the acrosome and flagellum are formed (Wu *et al.*, 2007). Its aberrant amount and distribution are correlated with the abnormal sperm head shape (Aarabi *et al.*, 2014). However, PAWP participation in the sperm head formation was not proven (Satouh *et al.*, 2015). The functional part of PAWP proteins is Pro-Pro-X-Tyr motif, interacting with the WW1 domain of other proteins involved in ubiquitin ligation, regulation of cell cycle, transcriptional activation (Kay *et al.*, 2000; Macias *et al.*, 2002). This is the main way in which PAWP attend oocyte fertilization. The PAWP role was identified in the meiosis resumption and pronucleus formation (Wu *et al.*, 2007; Aarabi *et al.*, 2014; Tavalaee *et al.*, 2017a). Oocyte activation was confirmed by microinjection of isolated PAWP from the post-acrosomal sheath, which initiate calcium efflux, exclusion of the polar body and maternal pronuclei formation (Wu *et al.*, 2007; Aarabi *et al.*, 2014; Satouh *et al.*, 2015). On the contrary, spermatozoa with missing PAWP are not capable of fertilization, which is also confirmed by a lower level of PAWP in spermatozoa of males with pathological sperm quality (Tavalaee and Nasr-Esfahani, 2016; Freour *et al.*, 2018; Tanhaei *et al.*, 2019).

PLCξ is another sperm protein indispensable for fertilization, with the main role in the calcium metabolism regulation in oocyte after fertilization (Sette *et al.*, 1997; Saunders *et al.*, 2002; Swann *et al.*, 2016). This mechanism was also evoked in metaphase II oocytes after PLCξ microinjection, and this was confirmation of its role in oocyte activation. PLCξ promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into the inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Yang *et al.*, 2013). This reaction starts the cascade of calcium releasing from endoplasmic reticulum and activates the pathway of protein kinase C (PKC) (Swann and Lai, 2016). Similar to PAWP, PLCξ is expressed less in spermatozoa with abnormal morphology, low motility, and/or high DNA fragmentation (Tavalaee *et al.*, 2017b; Azad *et al.*, 2018).

TR-KIT is expressed during spermatogenesis, but it is also present in ejaculated spermatozoa. In comparison to other SOAP proteins, apart from the post-acrosomal region, TR-KIT is localized in the mid-piece (Sette *et al.*, 1997, Sette *et al.*,2002; Muciaccia *et al.*,

2010). The role of TR-KIT was ascertained by its microinjection into the metaphase II oocytes and its activation accompanied by calcium efflux and induction of meiosis resumption (Sette *et al.*, 1997). Accordingly, SOAF proteins seem to be a useful negative marker of sperm quality, as well as their fertilization ability and embryo development potential.

Like in other cells, sperm centromere is composed of two centrioles - proximal (PC) and distal centriole (DC) and pericentriolar material (Vorobjev and Chentsov YuS, 1982). Altogether, they create major microtubule organizing center (MTOC), dealing with proper chromosome redistribution between two daughter cells (Sutovsky and Schatten, 2000). During spermatid elongation, PC used to be organized in the implantation fossa of the sperm head; DC identifies itself as the basement for flagellum growing. PC is only present in mature human spermatozoa, and thus it participates in the creation sperm aster after oocyte activation (Schatten, 1994; Manandhar *et al.*, 1998; Manandhar *et al.*,2005). This event is crucial for the approaching of maternal chromosomes, centriole duplication, and bipolar spindle organization. Followed by pronuclei fusion, the zygote is able to start the first cleavage (Navara *et al.*, 1994; Schatten, 1994; Chatzimeletiou *et al.*, 2008).

In particular, spermatozoa bring genetic information to the embryo that is regulated by epigenetic marks (Hammoud *et al.*, 2009; Siklenka *et al.*, 2015; Teperek *et al.*, 2016). Although most of the sperm chromatin is created by protamines, they are replaced by histones of maternal origin immediately after oocyte penetration (McLay and Clarke, 2003; Morgan *et al.*, 2005; Gaucher *et al.*, 2010). Nevertheless, the importance of proper chromatin condensation, protamine ratio and distribution of histone PTMs across the sperm genome is evident, detail information about the role of paternal histones in the embryo is still not clear (Brykczynska *et al.*, 2010; Gannon *et al.*, 2014; Steger and Balhorn, 2018; Cavé *et al.*, 2019). However residual histones and their PTMs are situated at developmentally important genes, transcription factors, miRNAs, and imprinted genes (Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010; Siklenka *et al.*, 2015; Pérez-Cerezales *et al.*, 2017; Wilson *et al.*, 2018; Lambrot *et al.*, 2019). Apparently, the most studied histone PTMs are methylated lysine 4, 9 and 27 of histone 3. Several chromatin immunoprecipitations were done, providing information about their potential role in the embryo development (Brykczynska *et al.*, 2010; Hammoud *et al.*, 2011; Kutchy *et al.*, 2017). Similar to DNA methylation, methylated histones mostly act as heterochromatin repressive marks. Accordingly, H3K27me is located in the promotors of a

suppressed gene. However, surprisingly, H3K4me used to be situated in the promoters of actively transcribed genes in the embryo. Promoters of imprinted genes are obviously marked by both repressive and enhancer marks (Brykczynska *et al.*, 2010). Furthermore, actively transcribed genes in the embryo are in sperm associated with H3K4me2 and H3K27me3, for example, transcriptional factors (OCT4, SOX2, NANOG, KLF4, and FOXD3) crucial for minor zygote activation, embryo genome activation and its cleavage (Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010). The existence of the paternal contribution in the embryo development was also proven by knockout and knockdown studies of enzymes responsible for the production of small non-coding RNAs, histone methylation and acetylation (Okada *et al.*, 2007; Bell *et al.*, 2014; Yuan *et al.*, 2016; Jiang *et al.*, 2018). Additionally, differences in DNA methylation, H4 acetylation, and H3 methylation were observed between fertile and non-fertile males (Schon *et al.*, 2018.; Jenkins and Carrell, 2011; Salas-Huetos *et al.*, 2014; Stuppia *et al.*, 2015; Kutchy *et al.*, 2017). Accordingly, we can assume that sperm epigenome participates in the regulation of embryo development.

Another sperm mechanism required for fertilization is the ubiquitin-proteasome system (UPS) (Sutovsky, 2003). This mechanism serves for recycling and degradation of waste proteins and organelles. Protein that should be removed is firstly marked by several ubiquitin molecules creating a chain that is recognized by 26S proteasome ensuring protein degradation (Glickman and Ciechanover, 2002; Sutovsky, 2011; Schreiber and Peter, 2014). UPS participates in sperm capacitation, but also in sperm oocytes interaction, where ubiquitin proteasome help in oocyte penetration via the degradation of *zona pellucida* proteins (Sutovsky *et al.*, 2004; Yi *et al.*, 2007a). Hence, UPS is indispensable for the degradation of paternal mitochondria (Song *et al.*, 2016). Whereas sperm mitochondria DNA is more sensitive to mutations, mitochondria are exclusively inherited from the maternal line. This process consists of prevention against lethal mutation trafficked by paternal mitochondria (Al Rawi *et al.*, 2011; Sutovsky, 2018).

In conclusion, spermatozoa significantly participate in healthy embryo development not just through their DNA, but also through proteins and other regulatory mechanisms. All of this is subject of study of paternal contribution in the embryo, but also a possible basement of infertility diagnostic and treatment.

1.7 Improvement of epigenome-based sperm selection for assisted reproductive therapy (ART)

From the microscope invention by Leuwenhoek, sperm selection is mainly ongoing based on the conventional sperm parameters involving motility and morphology (Wang and Swerdloff, 2014; Puerta Suárez *et al.*, 2018). However, this attitude to sperm selection bypasses many physiological barriers that are possibly also able to consider sperm epigenome (Suarez, 2016). Upcoming studies show decreased sperm quality, which was also displayed to the standards of the World Health Organization for human semen analysis. When we compare WHO 1999 to 2010 standards, the cut off values significantly decreased (concentration from 20mil/ml to 15mil/ml, morphology from 30% to 4% and total motility from 50% to 40%); therefore, fertile man according to WHO 2010 would be not considered fertile according to WHO 1999 (World health organisation, 1999; World Health Organization, 2010).

Apparently, spermatozoa quality is decreasing, and the most likely reason for this can be hidden in the sperm epigenome (Bracke *et al.*, 2018; Mishra *et al.*, 2018; Tang *et al.*, 2018; Rodprasert *et al.*, 2019). There are many studies confirming aberrant sperm epigenetic regulation as the cause of embryo development failures or offspring disorders (Jenkins and Carrell, 2011, 2012; Gannon *et al.*, 2014; Denomme *et al.*, 2017; Sun *et al.*, 2017; Ibrahim and Hotaling, 2018; Schon *et al.*, 2019). Considering this, the current massive use of ART bypassing inner molecular mechanisms is striking, and the necessity of studying the epigenetic impact and avoiding its harmful effect is crucial. Accordingly, the increase in 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 damaging them.

Microfluidic is the system of micro-channels, using the principles of female reproductive tract, like thermotaxis, chemical gradient, rheotaxis, tendency of spermatozoa swim near the boundary (Eamer *et al.*, 2015; Knowlton *et al.*, 2015; Suarez and Wu, 2016; Nosrati *et al.*, 2017). All of these principles show high efficiency in the selection the spermatozoa with better quality than other routinely used sperm selection techniques (Cho *et al.*, 2003; Shirota *et al.*, 2016). While the microfluidic system does not require centrifugation, this seems to be a better approach to the selection of spermatozoa without exposure to oxidative stress that has harmful effects on sperm quality (de Wagenaar *et al.*, 2015a; Wheeler

and Rubessa, 2016). The results of microfluidic spermatozoa selection correlate with improving spermatozoa motility, morphology and chromatin integrity, and thus significantly increase ART success (Matsuura *et al.*, 2013; de Wagenaar *et al.*, 2015b; Rappa *et al.*, 2016; Shirota *et al.*, 2016; Suarez and Wu, 2016; Wheeler and Rubessa, 2016). Whereas the relation of reactive oxygen species with chromatin integrity and changes in epigenome was proven (Elshal *et al.*, 2009; Iommiello *et al.*, 2015; Gunes *et al.*, 2018), we may expect that the use of microfluidic may be the tool to select spermatozoa without alternation in epigenome.

Nowadays, nanotechnologies are getting a lot of attention in different fields of medicine, and reproduction is no exception (Doane and Burda, 2012; Vasquez *et al.*, 2016). In sperm sorting, nanotechnologies receive special attention because they are able to interact with the appropriate surface protein that is already related to spermatozoa quality and has the potential to improve ART (Odhiambo *et al.*, 2011; Sutovsky *et al.*, 2015). Widely used nanotechnologies based sperm selection techniques is magnetic-activated cell sorting (MACS) that sort out apoptotic spermatozoa with the overexpression of annexin V (Degheidy *et al.*, 2015; Stimpfel *et al.*, 2018). In 2001, method called sperm ubiqutin taq assay (SUTI) was established, which uses ubiqutin as natural marker of defective spermatozoa (Sutovsky, 2001; Baska *et al.*, 2008). The spermatozoa selected by SUTI have better motility, morphology and showed higher potential in ART. Furthermore, SUTI was able to improve the fertilization rate in smokers whose spermatozoa are characteristic by aberrant DNA methylation and reactive oxygen species (Muratori *et al.*, 2005; Ozanon *et al.*, 2005; Varum *et al.*, 2007; Eskandari-Shahraki *et al.*, 2013).

Raman spectroscopy is a non-invasive chemical method providing detailed information about the chemical structure of molecules without destruction of the study subject (Huang *et al.*, 2014). Recently, Raman spectroscopy has begun to be used in andrology (Štiavnická *et al.*, 2017). Utilizing this method, different sperm regions such as acrosome, head, flagellum were distinguished. Furthermore, the method was used for differentiation of X and Y spermatozoa as well as detection of DNA damage (Sánchez *et al.*, 2012; De Luca *et al.*, 2014; Huang *et al.*, 2014; Liu *et al.*, 2014; Mallidis *et al.*, 2014). Apart from this, studies done on human Jurkat cells proved the ability to distinguish histone acetylation (Poplineau *et al.*, 2011). According to the study, Raman spectroscopy seems to be promising non-invasive sperm selection technique with potential to distinguish epigenome aberration (Fig. 4).

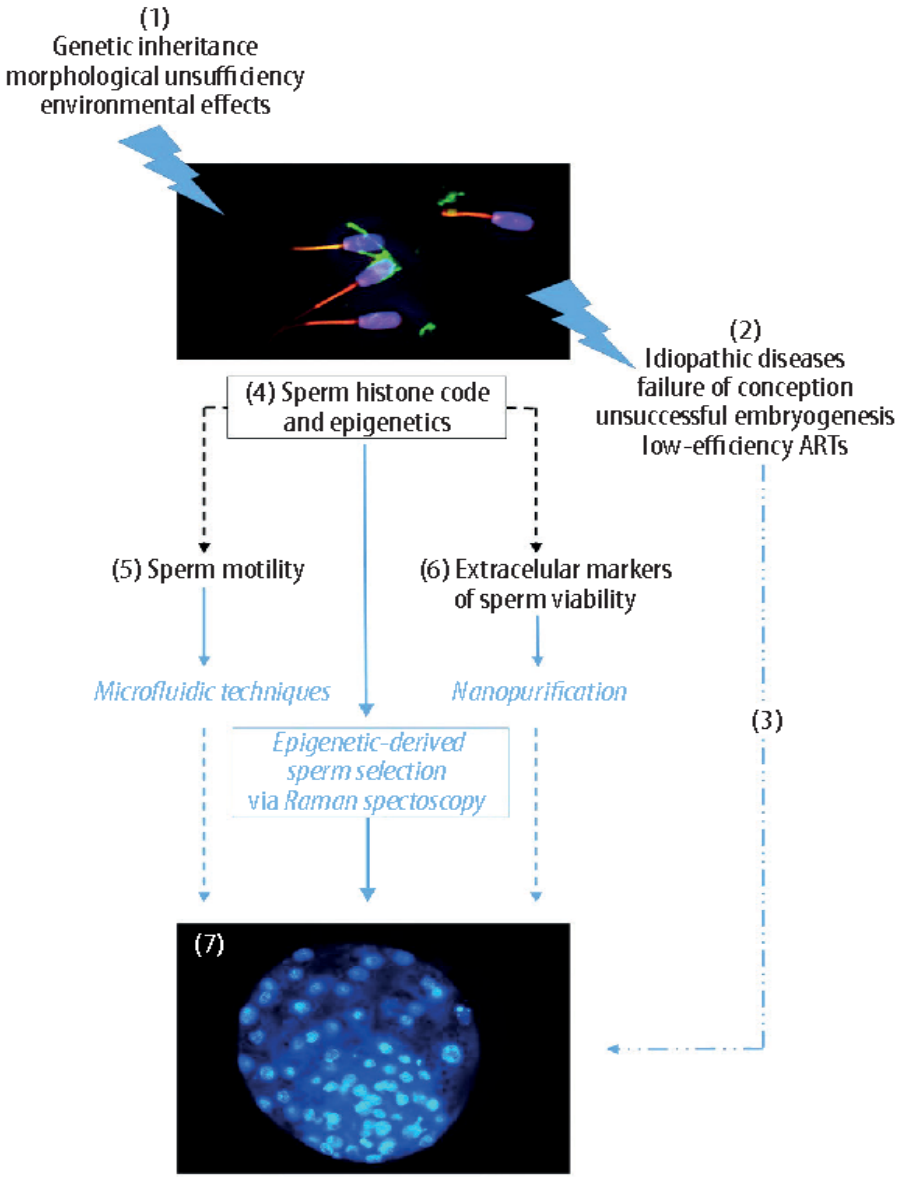

Figure 4. Application of Raman spectrometry in sperm selection for ART (Štiavnická *et al.*, 2017)

There are many other known sperm quality markers, especially sperm borne oocyte activating factors with proven relation to fertility and potential to improve ART (Sutovsky *et al.*, 2015; Zhong *et al.*, 2015; Tavalaee and Nasr-Esfahani, 2016). However, the necessity to observe their non-invasive application in the sperm selection process is crucial. Thus,

subsequent research should investigate the relation of possible new sperm quality markers with already settled non-invasive sperm selection techniques to verify their reliability and clarify their possible application in ART, which could improve fertilization rate, but also healthy embryo development and decrease the controversial harmful effect on future offspring born by ART.

2 HYPOTHESIS AND AIMS

Nowadays, around 15% of couples suffer from some kind of infertility with equal gender contribution. We can find the reason for this in our lifespan and postponing of parenthood to a later age. This creates quite a long time period for the exposition of the germ cells to the negative effect of the environment, leading to aberrant epigenome and thus increase infertility. Accordingly, 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. 5).

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.

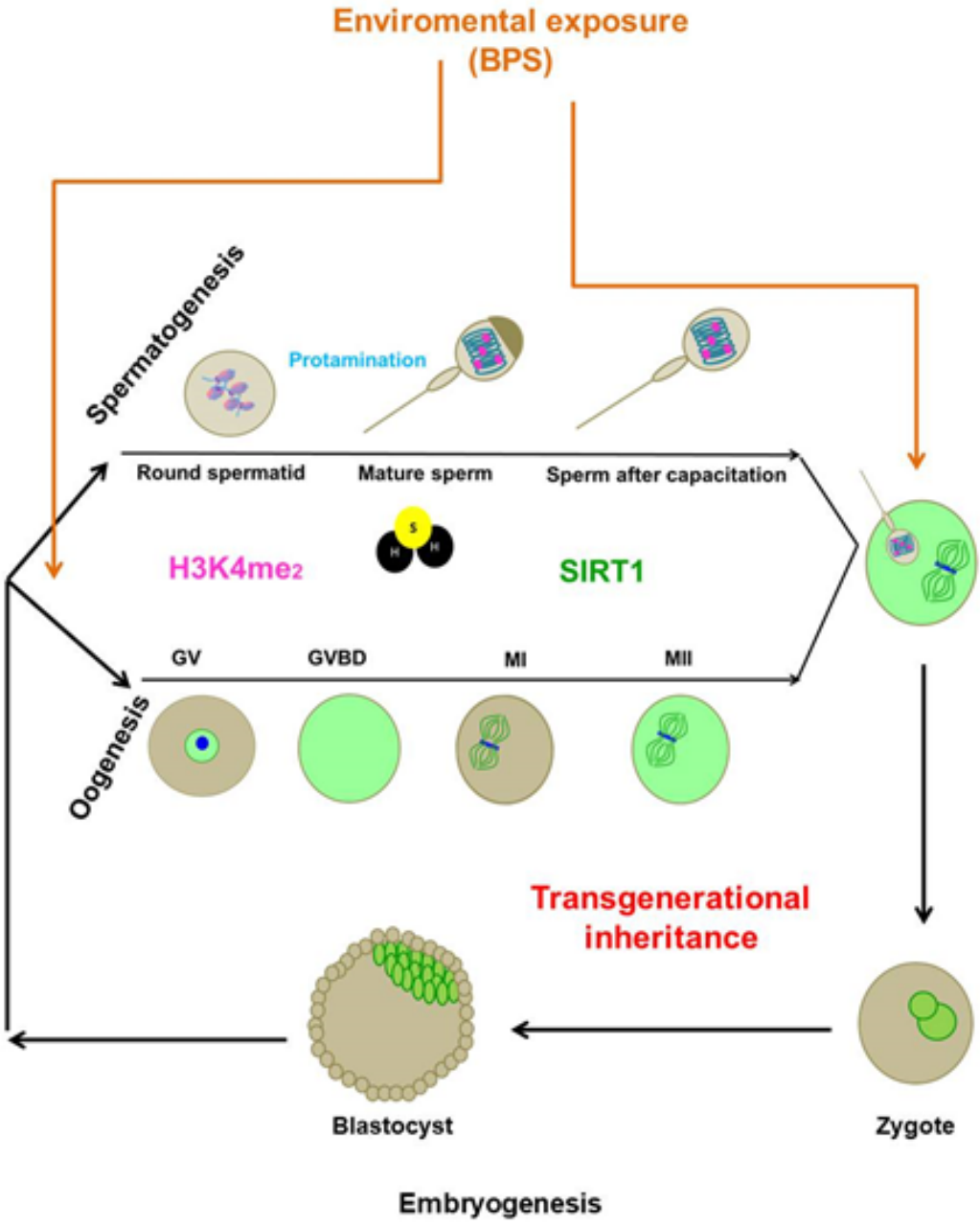

Figure 5. **Diagram describing the hypothesis and further implications**. The diagram displayed the most important events of gamete development, leading to the fertilization. On the one side it is protamination through the spermatogenesis that also involves H3K4me2. If the protamination is not completed, ejaculated spermatozoa display decreased quality as well as excess of H3K4me2. This event could be indirectly regulated by SIRT1, showing relocation to ooplasm of metaphase MII oocytes during oocytes maturation. Thereafter, oocytes are ready for the fertilization. For achievement of fertilization ability of the sperm, the

capacitation is strictly required; this is possibly regulated by gasotansmitter H_2S through posttranslational modifications of proteins involved in that process. After fertilization in the first few hours, paternal mitochondria are degraded; paternal centriole participates in the chromosome organization and approaching of pronuclei to fuse. Sperm and oocyte 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. For simulation of these events, bisphenol S (BPS) has been choiced for mice-based experiments.

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 of the 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 World Health Organization standards (WHO 2010). Sperm concentration and motility were evaluated subjectively using Makler chamber, and total and progressive motility were assessed.

Accordingly, semen samples were classified based on semen quality into three groups:

1. normozoospermic – samples with a concentration above 15 mil/ml and motility above 32%
2. asthenozoospermic – samples with progressive motility under 32%
3. oligoasthenozoospermic – samples with progressive motility under 32% and concentration below 15 million per milliliter (mil/ml).

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 adult (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.

Females were administered with i.p. 5 IU PMSG and sacrificed 48 hours later. Ovaries were dissected and immature fully-grown oocytes at the GV stage were isolated and manipulated in M2 medium supplemented with 100 µmol/L isobutyl-methylxanthine (IBMX). Fully-grown and cumulus cell-free GV oocytes with intact ooplasm were placed into M16 medium containing 100 µmol/L IBMX for 1 h, followed by *in vitro* maturation in IBMX-free M16 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, and cumulus cells were removed using incubation with 0.1% bovine testicular hyaluronidase for 5 min.

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 using a hemocytometer (ThermoFisher Scientific) and ranged from 250 to 350 million/ml. Only ejaculates with >80% motility were used. Sperm rich fraction was used for all analyses, except analysis during in vitro capacitation (IVC).

3.4 Analysis of mitochondria activity and early apoptosis of sperm

The aim of that analysis was characterization of spermatozoa by mitochondria activity membrane integrity. We have detected mitochondria membrane potential in combination with the signs of early apoptotic cells using flow cytometry.

For the detection of mitochondrial activity and early apoptotic spermatozoa, sperm samples were incubated for 30 minutes at room temperature with the staining solution of 100nM MitoTracker Deep Red (Thermo Fisher Scientific, Waltham, MA, USA) and 50nM YO-PRO1 (Thermo Fisher Scientific, Waltham, MA, USA) in Biggers-Whitten Whittingham medium (BWW), prepared according to the recipe (Biggers *et al.*, 1971). Acquisition was performed on a FACSVerse™ flow cytometer (Becton Dickinson, San Diego, USA) using BD FACSuite™ software (Becton Dickinson, San Diego, USA). Data was collected from 5000 cells. While excitation for YO-PRO1 was carried out with a 488 nm laser, MitoTracker Deep Red was excited at 640 nm. For YO-PRO1 green fluorescence, a 537/32BP filter was used and MitoTracker Deep Red was detected by a 586/42BP filter. MitoTracker+/YO-PRO1- spermatozoa were considered viable with active mitochondria, and MitoTracker-/YO-PRO1+ as apoptotic spermatozoa with non-functional mitochondria. Flow cytometry data was analyzed using WEASEL software Ver. 3 (WEHI, Melbourne, Australia).

3.5 Sperm chromatin structure assay (SCSA®)

Sperm chromatin structure assay was performed according to the protocol described by Evenson and Jost (2001). The assay is based on the acid based in situ denaturation with subsequent staining by metachromatic acridine orange. Single stranded DNA equivalent to fragmented, fluorescence in red, while double stranded untouched emits green fluorescence. The output of the analysis is DNA fragmentation index (%DFI) and high DNA stainability (%HDS). While %DFI is numbered like percentage of red fluorescence to total fluorescence, %HDS is defined by excess of green fluorescence caused by approximately 5 times less chromatin condensation corresponding to round spermatids.

Samples were diluted with 250 µl TNE buffer (0.15 M NaCl; 0.01 M Tris HCl; 1 mM EDTA; pH 7.4) at a concentration of 2mil/ml and immediately frozen in liquid nitrogen. On the day of analysis, 200 µl of the thawed sample were incubated with acid detergent (0.08 M HCl; 0.15 M NaCl; 0.1% Triton-X-100; pH 1,4) for 30 seconds; subsequently 1.2 ml of acridine

orange solution (0.037 M citric acid; 0.126 M Na_2HPO_4; 0.0011 M dissodium EDTA; 0.15 M NaCl; pH 6,0) was added and incubated for 2 minutes. Immediately after, the samples were run by FACSVerse^{TM} flow cytometer, controlled with BD FACSuite™ software. Data was collected from 5000 cells. Excitation of acridine orange was carried out with a blue laser (488 nm), while green fluorescence was detected by a 537/32BP filter, and red fluorescence was assessed by the 700/54BP. Each sample was run twice, and data was analyzed using WEASEL software Ver. 3 (WEHI, Melbourne, Australia).

According to the results, samples were divided based of %HDS into two groups: 1) low HDS – %HDS≤15, and 2) high HDS – %HDS>15.

3.6 Histone detection by flow cytometry

For histones detection, sample preparation and histone analysis by flow cytometry was performed according to Li *et al.* ref. For the detection of H3K4me2, polyclonal rabbit anti-H3K4me2 (1:100, Abcam, Cambridge, UK) were used, and Alexa Fluor 488 conjugated goat anti-rabbit secondary antibody (1:200, Abcam, Cambridge, UK). Acquisition was performed on a FACSVerseTM flow cytometer using BD FACSuite™ software. Data was collected from 5000 events. Blue laser (488 nm) was used and fluorescence signal was collected by filter 537/32BP for the excitation of Alexa Fluor 488. Isotype control was performed for each sample, and mean fluorescence intensity was measured in the sample and isotype control. Data was analyzed using WEASEL software Ver. 3 (WEHI, Melbourne, Australia), and the final value of fluorescence intensity was obtained after subtracting mean fluorescence intensity of isotype control from the signal of the sample.

3.7 Western blot analysis of sperm and oocytes

The samples, either cells or tissue, were subjected to western blotting. Samples of mouse or human semen were washed twice with PBS, and subsequently dissolved in lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 120mM DTT, 40 mM TRIS base), and were then solubilized with the Laemmli buffer (0.003% Triton-X-100 and 0.001% SDS), enriched with Complete Mini Protease Inhibitor Cocktail (Roche, Switzerland). Mouse oocytes were collected and lysed in Laemmli buffer, as aforementioned, with subsequent boiling for 5 minutes. Thereafter, the samples were subjected to SDS-PAGE electrophoresis in precast

gradient gels (4 – 12.5%) and blotted using the Trans-Blot Turbo™ Transfer System (Biorad Laboratories, Steenvoorde, France) onto a PVDF membrane (GE Healthcare Life Sciences, Amersham, UK). The membrane was then blocked in 1% BSA in TBS with 0.5% Tween-20 (TBS-T) for 60 min at room temperature. Subsequently, the membrane was 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; Sigma-Aldrich, St. Louis, USA) overnight at 4°C. Furthermore, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG in TBS-T (1:10.000; Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. Proteins with adequate molecular weight were detected using the ECL Select Western Blotting Detection Reagent (GE Healthcare Life 1Sciences, Amersham, UK) and visualised by the ChemiDoc™ MP System (Bio-Rad Laboratories, Steenvoorde, France).

3.8 Immunocytochemistry, microscopy and image analysis

Sperm samples were washed with PBS, placed onto microscopy slides and allowed to dry. Spermatozoa on slides were fixed with 4% of paraformaldehyde for 15 minutes and washed with PBS. They were then permeabilized with 0.5% Triton-X-100 and blocked with a solution of 5% normal goat serum (NGS) with 0.1% Triton-X-100 in PBS for 1 hour at room temperature. Subsequently, spermatozoa were incubated with a rabbit polyclonal anti-H3K4me2 antibody (1:100; Abcam, Cambridge, UK) for 1 hour at room temperature, washed twice with PBS and incubated with a AlexaFluor 488 conjugated goat anti-rabbit secondary antibody (1:200). Finally, the sperm samples were washed twice and mounted onto slides in a VectaShield medium with 4'6'-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA).

Oocytes at all stages were fixed in 4% paraformaldehyde in PBS with 0.1% polyvinyl-alcohol (PVA) for 30 min. Subsequently, all oocytes were permeabilised in PBS containing 0.04% Triton-X-100 and 0.3% Tween 20, for 15 min. Thereafter, oocytes were blocked in 1% BSA in PBS with Tween 20 for 15 min. The 1h incubation of oocytes with specific antibodies: anti-SIRT1 (1:200; Abcam, Cambridge, UK), anti-α tubulin (1:200; Cell Signaling Technology, Leiden, Netherlands), anti-acetylated α-tubulin (1:200; Abcam, Cambridge, UK), anti-H3K9me2/3 (1:200; Abcam, Cambridge, UK), anti-H3K4me2 (1:200; Abcam,

Cambridge, UK), and anti-H2AK119ub (1:200; Cell Signaling Technology, Massachusetts, USA), H3K27me2 (1:200; Abcam, Cambridge, UK) and anti-5′-methyl cytosine (1:200; Abcam, Cambridge, UK). Thereafter, washing and 1h incubation with a cocktail of anti-mouse-AlexaFluor 488 and anti-rabbit-AlexaFluor 647 (1:200), respectively, were used. Concurrently with the washing, after the cocktail of secondary antibodies, phalloidin (1:200; Thermo Fisher Scientific, Waltham, MA, USA) was applied for 15 min for β-actin visualisation. Stained oocytes were mounted onto slides in a Vectashield medium with 4'6'-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA). Images were acquired using spinning disk confocal microscope Olympus IX83 (Olympus, Germany) and VisiView® software (Visitron Systems GmbH, Germany).

Immuno-stained oocytes were subjected to measurement of integrated density (expressing signal intensity) of appropriate colour channels using ImageJ software (NIH, Bethesda, CA, USA). Nuclear signal intensities were scaled by the signal intensity of corresponding ooplasms. Thereafter, the values of integrated density were related to control oocytes (VC = 1). The JACoP (Just Another Co-localisation Plugin) approach for colocalisation of SIRT1 with spindle α-tubulin was used. The Costes' randomisation (Costes' rand), modifying Pearson's coefficient Rr according to Costes et al. (2004), and Manders' overlap coefficients (R, M1 and M2) were used for estimation of colocalisation and overlap. Colocalisation analysis was performed on oocyte spindles used as the region of interest (ROI).

3.9 Quantification of H_2S-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-X-100 and 5% NGS for 40 minutes. Afterward, spermatozoa were incubated with primary antibody rabbit polyclonal anti-CBS (1:100; Abcam, Cambridge, UK), anti-CTH (1:100; Abcam), anti-MPST (1:50; Abcam) at 4 °C during the night. After two washings, incubation with secondary antibody Alexa fluor 488 (1:200; Abcam), Hoechst 33342 (1:1500, Thermo Fisher Scientific) and lectin PNA from Arachis hypogaea conjugated with Alexa fluor 647 (1:2500, PNA-AF647, Thermo Fisher

Scientific) was done for 40 minutes. The spermatozoa were then washed, and signal intensity was quantified using Amnis flow cytometry.

3.10 H_2S donor treatment of ejaculate spermatozoa

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; 100uM), 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, PI, Thermo Fisher Scientific, Waltham, MA, USA) and FZ3 (1:1500, Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes. The samples were subsequently spun down and re-suspended in NCM, followed by measurement using Amnis Flow cytometry.

3.11 *In vitro* capacitation with the presence of H_2S donor

Semen samples were washed of seminal plasma once with non-capacitating media (NCM) and subsequently 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 a concentration of H_2S donor and according to previous observation. During 4 hours of IVC, the sperm were treated with 100 uM NaHS. Distilled water was used as vehicle control. The capacitation status was measured each hour using Amnis flow cytometry with the same fluorescent probes and aforementioned protocol for ejaculated spermatozoa.

3.12 Acquisition of Amnis Flow cytometry

The acquisition for H_2S-releasing enzymes in ejaculated and capacitated spermatozoa was carried out by INSPIRE® FS software. Excitation of Hoechst 33342 was assessed by

laser 405 nm with a power of 10 mW, Alexa fluor 488 by laser 488 nm and power of 40 mW, PNA-AF647 by laser 642 nm and power 20 mW.

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. INSPIRE® FS software was applied for the acquisition. Excitation of Hoechst 33342 was carried out by laser 405 nm with a power of 10 mW, FZ3 by laser 488 nm and power of 60 mW, PI by laser 561 nm and power of 40 mW, PNA-AF647 by laser 642 nm and power of 25 mW. The detailed information are referred by Kerns *et al.* (2018).

3.13 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

Sperm epigenetic has deserved attention in recent years (Brykczynska *et al.*, 2010; Verma *et al.*, 2015; Luense *et al.*, 2016; Lambrot *et al.*, 2019). Association of aberration in sperm epigenome with decrease spermatozoa quality and success of ART was proven (Francis *et al.*, 2014; Belva *et al.*, 2016; Lou *et al.*, 2018). However, there is still information missing explaining their role. Moreover, improper chromatin condensation caused by a defect in protamination has proven effect on DNA accessibility to be damaged (Castillo *et al.*, 2011; Simon *et al.*, 2011). This study showed the association of chromatin immaturity with routinely assessed sperm parameters, that were displayed to the excess of dimethylated lysine K4 of histone H3 (H3K4me2). While H3K4me2 is associated with active transcription and this chromatin relaxation, some studies found its higher expression during protamination that predict H3K4me2 as a promising indicator of chromatin immaturity.

Observation of this association can help in the prediction of ART success and verification of sperm quality after already established non-invasive spermatozoa selection techniques for ART. Therefore, the aim was to estimate H3K4me2 in spermatozoa with different quality and chromatin maturity assessed.

Characterisation of spermatozoa with different quality

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, the semen samples were divided into three groups; normozoospermic (N), asthenozoospermic (A) and oligoasthenozoospermic (OA), and compared amongst themselves in assessed parameters (Table 1). 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® (Table 2) as referred Evenson and Jost (2000).

Table 1. Sample characterization according to the semen quality. Values are expressed as the median (quartiles). Differing letter superscript in the same row denotes significant differences (*p<0.05*). The number of patients in the N, A and OA groups is indicated in the heading of the table.

	N (22)	A (63)	OA (14)	*p* value
Age	34.00(31.00-37.00)[ab]	36.00(33.00-40.00)[a]	32.50 (31.00-37.00)[b]	<0.05
Volume (ml)	2.70(2.10-3.40)[a]	3.15(2.50-4.20)[a]	3.50(2.40-5.10)[a]	0.139
Concentration (mil/ml)	84.00(69.00-109.00)[a]	49.00(29.00-78.00)[b]	7.00(4.00-10.00)[c]	<0.001
Total motility (%)	66.00(64.00-73.00)[a]	49.00(37.00-56.00)[b]	42.00(36.00-56.00)[b]	<0.001
Progressive motility (%)	37.50(30.00-50.00)[a]	8.00 (3.00-14.00)[b]	0.00 (0.00-10.00)[b]	<0.001
MitoTracker+/YO-PRO1-	71.31 (63.65-84.33) [a]	53.94 (43.50-66.37) [b]	51.10 (38.95-57.94) [b]	<0.01
MitoTracker-/YO-PRO1+	14.05 (9.76-18.41) [a]	22.78 (17.18-30.89) [b]	24.17 (20.58-30.47) [b]	<0.01
% DFI	7.73 (5.39-11.70) [a]	12.89 (9.18-19.31) [b]	18.55 (12.54-25.90) [b]	<0.01
% HDS	6.87 (3,98-10,09) [a]	10.43 (6.63-18.32)[a]	11.77 (5.52-30.38)[a]	0.044
H3K4me2	434.80 (233.90-731.90) [a]	651.10 (316.30-1197.80) [a]	2185.80 (790.60-4049.80) [b]	<0.01

Tables 2. Sample characterization according to the HDS. Values are expressed as the median (quartiles). Differing letter superscript in the same row denotes significant differences ($p < 0.05$). The number of patients the group with low and high HDS is indicated in the heading of the table.

	%HDS≤15 (69)	%HDS>15 (30)	*p* value
Age	36.00 (32.00-39.00)[a]	34.50 (31.00-39.00) [a]	0.278
Volume (ml)	3.30 (2.40-4.50) [a]	3.00 (2.50-3.20) [a]	0.101
Concentration (mil/ml)	57.00 (29.00-86.00) [a]	31.50 (18.00-70.00) [b]	<0.05
Total motility (%)	52.00 (42.00-65.00) [a]	49.00 (33.00-61.00) [b]	<0.05
Progressive motility (%)	11.00 (5.00-24.00) [a]	6.00 (0.00-16.00) [b]	<0.05
MitoTracker+/YO-PRO1- (%)	61.00 (49.50-71.23) [a]	52.45 (39.50-66.92) [a]	0.067
MitoTracker-/YO-PRO1+ (%)	19.29 (14.18-28.90) [a]	23.33 (18.49-34.52) [a]	0.180
%DFI	11.59 (7.35-18.41) [a]	13.45 (11.03-19.31) [a]	0.124
H3K4me2	479.20 (280.00-1190.70) [a]	813.40 (581.500-1670.00) [b]	<0.01

Pathological spermiograms have less active mitochondria

The aim of this analysis was to compare mitochondrial membrane potential (MitoTracker+/-) and early apoptotic cells (YO-PRO1+/-) between N, A and OA sperm samples. We found a significantly higher percentage of spermatozoa with active mitochondria (MitoTracker+/YO-PRO1-) in N samples compared to A and OA samples. Inversely, the percentage of MitoTracker-/YO-PRO1+ spermatozoa was significantly higher in the A and OA samples compared to the N-group (Table 1, Fig. 6).

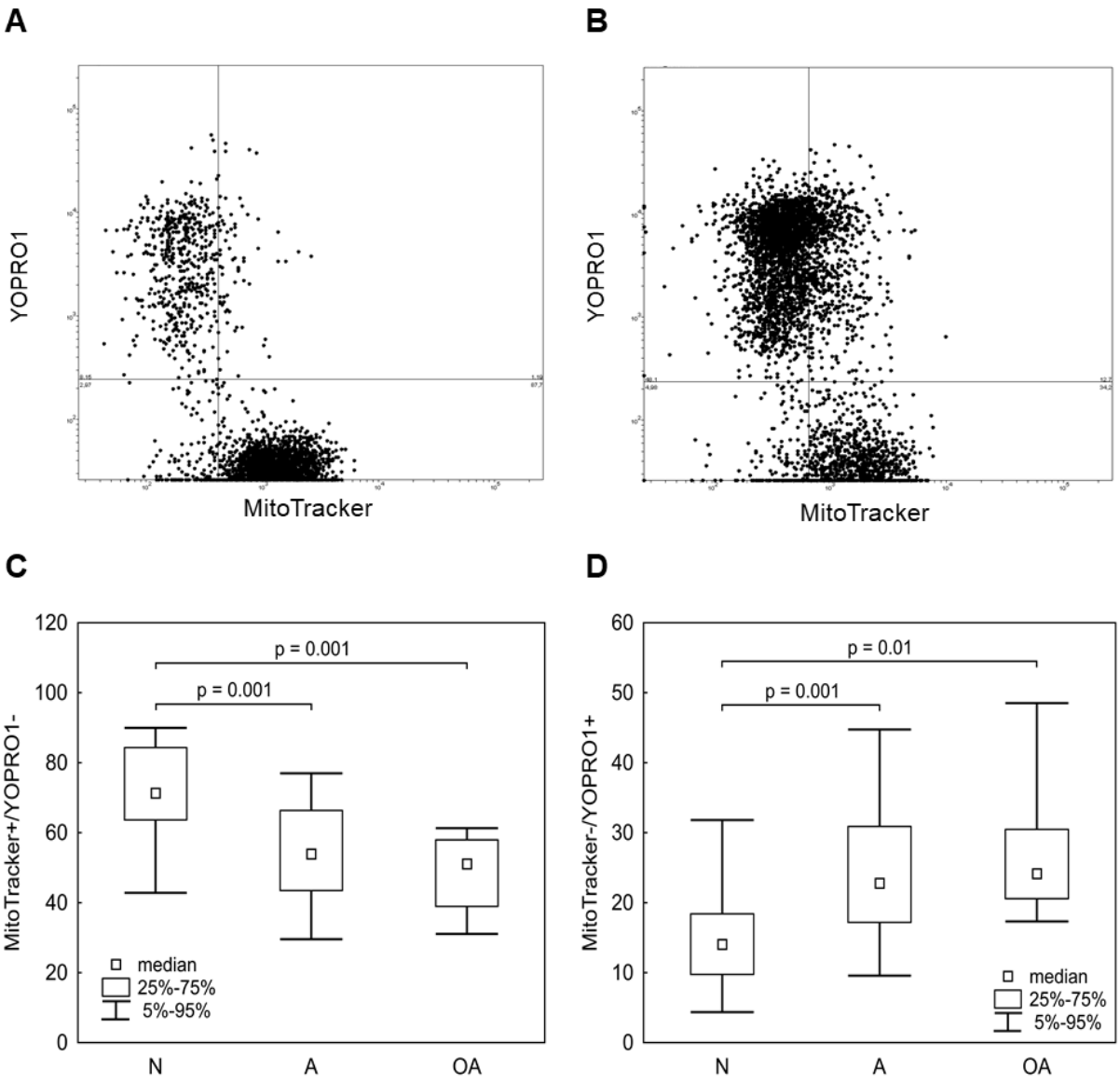

Figure 6. The relation sperm mitochondrial activity and membrane integrity to sperm quality. (A) Representative scatter plots of a sample with high mitochondrial membrane potential **(B)** and a sample with high percentage of non-viable spermatozoa. **(C)** Average percentages of MitoTracker+/YO-PRO1- **(D)** and MitoTracker-/YO-PRO1+ spermatozoa in the normozoospermic, asthenozoospermic and oligoasthenozoospermic samples. Data are expressed as the median with appropriate quartiles and different superscripts indicate statistical significance ($p < 0.05$).

Immature spermatozoa involve more DNA fragmentation

The chromatin integrity of the cohort was characterized by SCSA®, assessing both %DFI and %HDS (Evenson and Jost, 2000). 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 (Fig. 7). However, a significant trend (Spearman's correlation: p=0.021) of increasing %HDS was proven for decreasing level of spermiogram quality (N > A > OA).

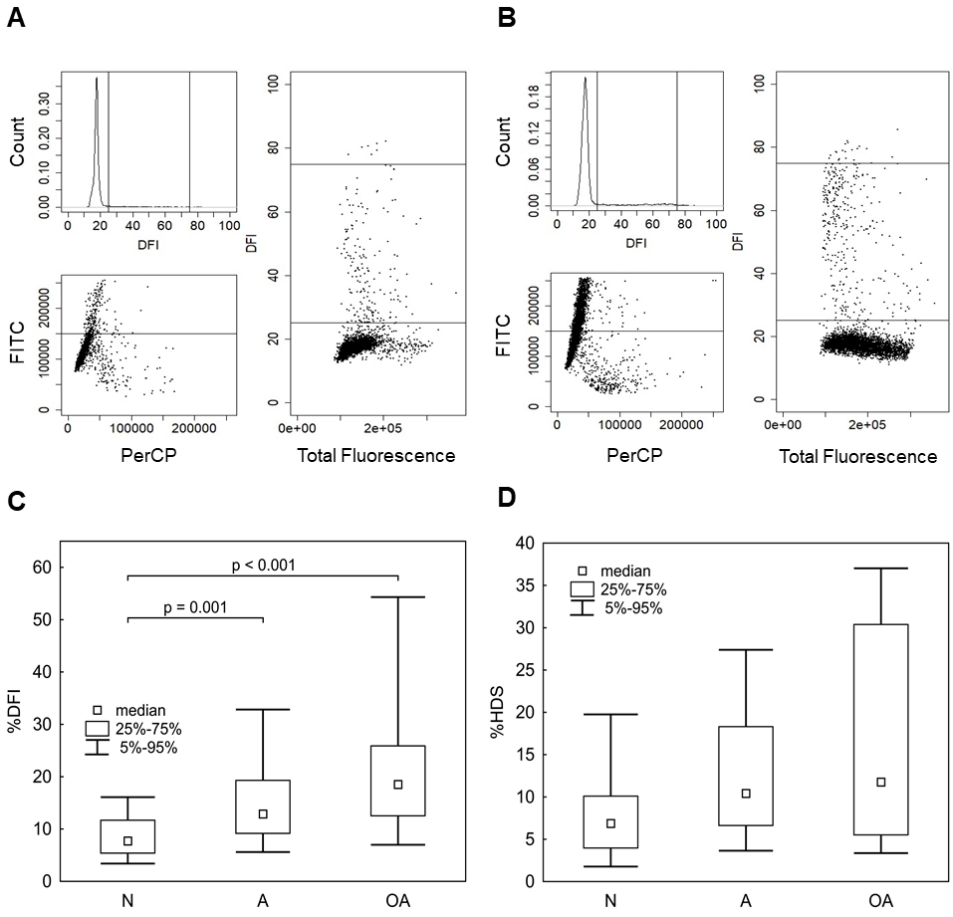

Figure 7. The relationship of semen quality with DNA fragmentation and chromatin maturity measured by SCSA®. (A) Representative histograms and scatter diagrams of the SCSA® analysis in sample with HDS≤15 **(B)** and sample with HDS>15. **(C)** Comparison of %DFI **(D)** and %HDS between normozoospermic, asthenozoospermic and

oligoasthenozoospermic samples. The data are expressed as the median and appropriate quartiles, and different superscripts indicate statistical significance ($p < 0.05$).

Moreover, the %HDS correlated positively with %DFI and negatively with sperm concentration, and with total and progressive motility. We also found a negative correlation between the percentage of spermatozoa with active mitochondria (MitoTracker+/YO-PRO1-) and %HDS (Table 3).

Table 3. Spearman's Rank correlation test of %HDS and other sperm parameters Correlations are considered significant at $p < 0.05$.

	% HDS	***p* value**
Age	-0.125	0.220
Volume (ml)	-0.072	0.480
Concentration (mil/ml)	-0.331	<0.001
Total motility (%)	-0.270	<0.001
Progressive motility (%)	-0.310	<0.001
MitoTracker+/YO-PR O1- (%)	-0.261	<0.05
MitoTracker-/YO-PRO1+ (%)	0.171	0.140
%DFI	0.200	<0.05

H3K4me2 relation to sperm maturity

The goal of this experiment was to detect the H3K4me2 modification by flow cytometry in sperm samples of different quality %HDS. We confirmed the specificity of the H3K4me2 antibody and its localization to the nucleus by western blotting and immunofluorescence (Fig. 8,9). Our results show 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 the N samples (Fig. 10). Similarly, significantly higher H3K4me2 levels were detected in the group with %HDS>15 (Table 2). Additionally, H3K4me2 levels were correlated positively with %HDS, but negatively with sperm concentration, progressive motility (Table 4).

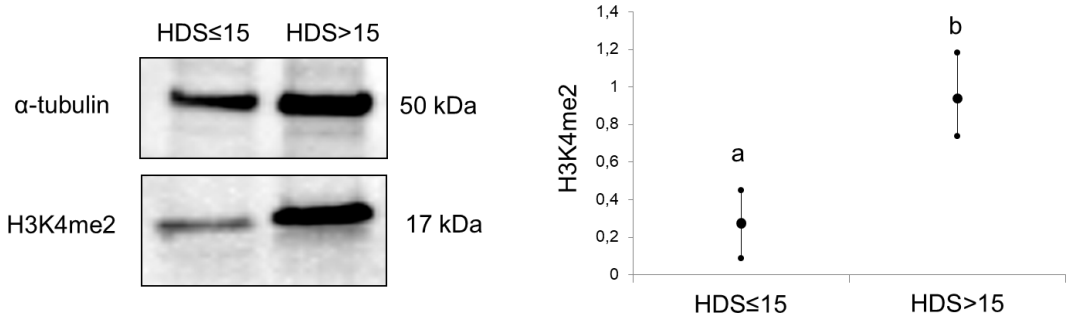

Figure 8. Representative picture of H3K4me2 western blot detection. The difference in H3K4me2 abundance in a high vs. low HDS sample, demonstrated by Westen blot densitometry, with anti-alpha tubulin antibody as a loading control. The data are expressed as the mean including min-max whiskers, and different superscripts indicate statistical significance ($p < 0.05$). HDS, high DNA stainability index

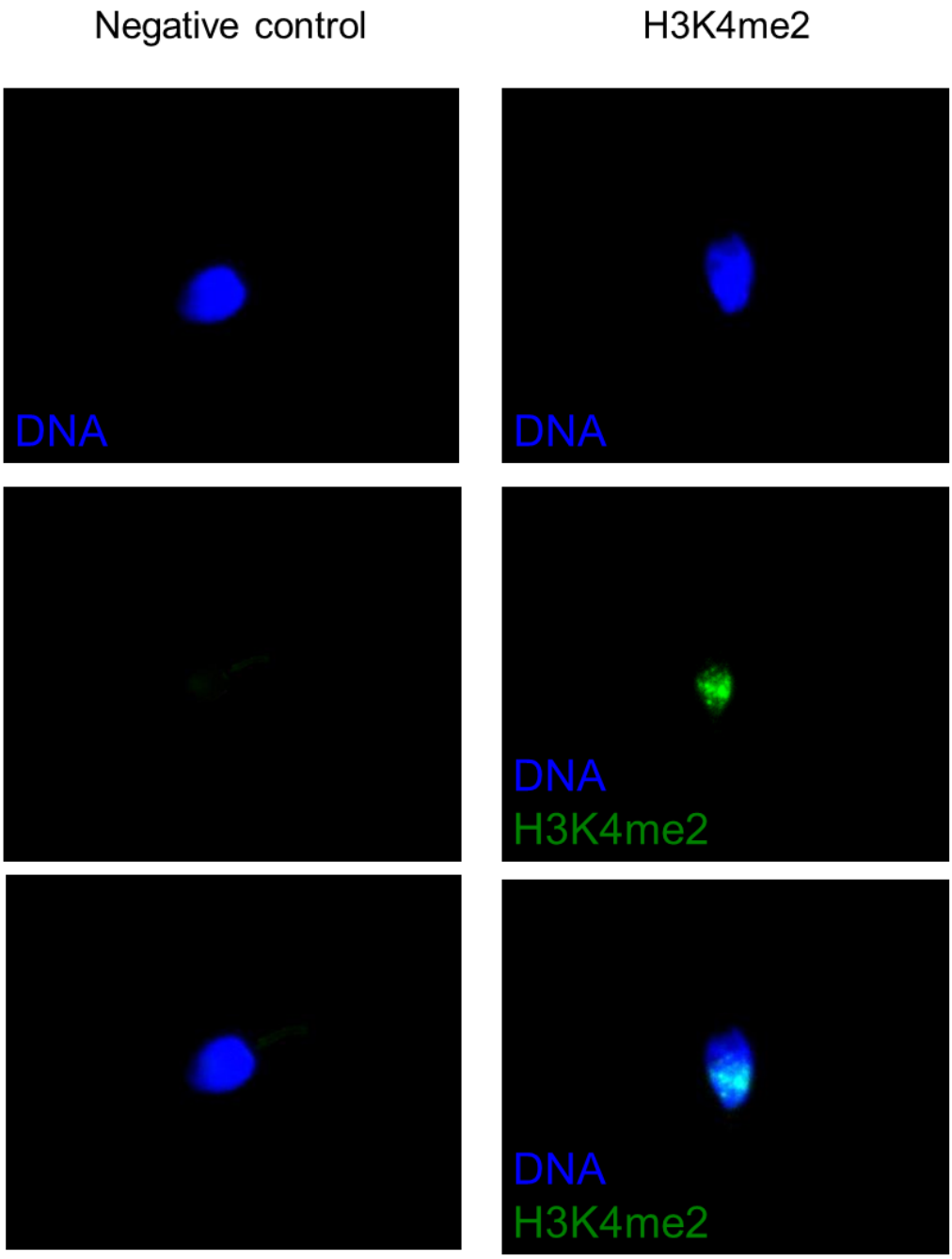

Figure 9. Representative pictures of sperm subcellular localization of H3K4me2. Sperm head shows the most signal of anti-H3K4me2 antibody binding. Normospermic (N) semen sample was used for immunocytochemistry. Negative control was prepared by omitting anti-H3K4me2 antibody.

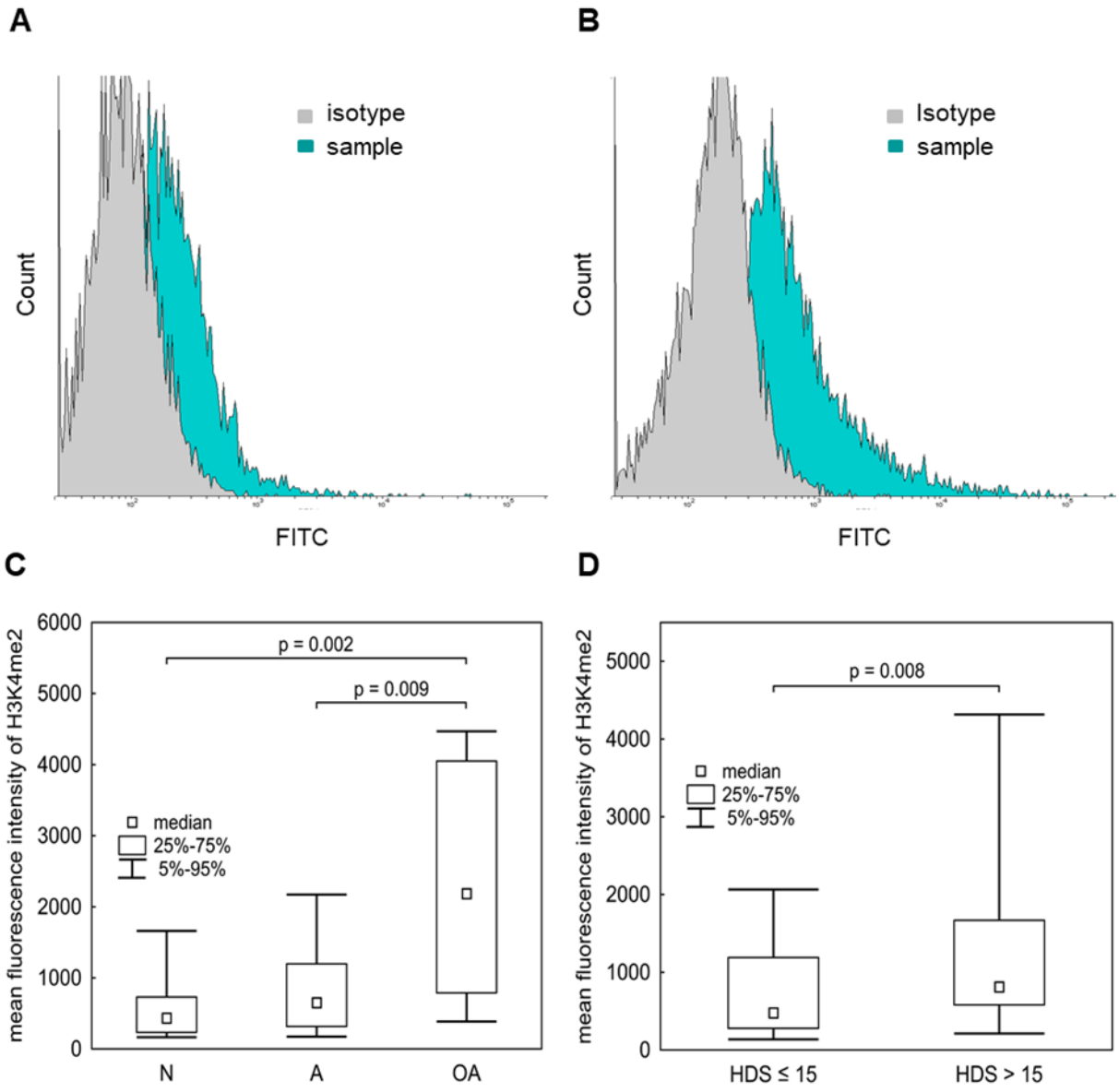

Figure 10. Relationship of H3K4me2 labelling with semen quality and chromatin maturity. (A) Representative images of H3K4me2 histograms generated by flow cytometry, including a normospermic sample with HDS≤15 **(B)** and a sample with pathological semen quality and HDS>15. **(C)** The comparison of H3K4me2 fluorescence intensity between normozoospermic, asthenozoospermic, oligoasthenozoospermic samples **(D)** and between samples with HDS≤15 and HDS>15. The data are expressed as the median and appropriate quartiles, and different superscripts display statistical significance ($p<0.05$).

Table 4. Spearman's Rank correlation test of H3K4me2 and other sperm parameters. Correlations are considered significant at $p < 0.05$.

	H3K4me2	*p* value
Age	-0.120	0.261
Volume (ml)	0.120	0.260
Concentration (mil/ml)	-0.570	<0.01
Total motility (%)	-0.093	0.374
Progressive motility (%)	-0.250	<0.05
MitoTracker+/YO-PRO1- (%)	-0.175	0.140
MitoTracker-/YO-PRO1+ (%)	0.010	0.410
% DFI	0.162	0.120
% HDS	0,470	<0.01

These observations have been published in a journal with an impact factor and are attached as the 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. 2019. *Systems Biology in Reproductive Medicine*. 2019. Under review (**IF_{2017}=1.582**)

4.2 Up-regulation of epigenetic code in sperm and oocytes

S-sulfhydration as one of possible protein posttranslational modifications can modify sperm proteins function and thus make epigenetic and non-epigenetic changes. Moreover, the machinery of histone deacetylases is considered: i) histone deacetylase S-sulfhydration is a known PTM, but the role is still undescribed, and ii) NAD^+-dependent histone deacetylases have a wide range of histone and non-histone targets, required in gametogenesis as well as embryonic development.

4.2.1 The involvement of H_2S in sperm capacitation

Up today, there are studies describing the role of gasotransmitters, especially nitric oxide (NO) and hydrogen sulphide (H_2S) in male reproduction (Nevoral *et al.*, 2016). Moreover, there are indications leading to possible regulation of capacitation by gasotransmitters (Wang *et al.*, 2018; Lefièvre *et al.*, 2007). These small gaseous molecules, namely H_2S, NO and carbon monoxide (CO), endogenously produced in various cells and tissue, play an important role in reproduction. The most studied gasotransmitter in the male reproduction is NO that was proven as a signalling pro-capacitating molecule, produced by oocyte cumulus cells and oviduct of female reproductive tract (Machado-Oliveira *et al.*, 2008; Lefièvre *et al.*, 2009).

Our study showed the inhibition of sperm capacitation by H_2S. The preliminary data related to the study were achieved during fellow at the University of Missouri, with the kind help and supervision of prof. Peter Sutovsky, Dr.h.c. Currently; the data are under preparation for manuscript submission to a journal with an impact factor.

Capacitated spermatozoa express less H_2S-releasing enzymes

We have detected all three H_2S-releasing enzymes (Cystahionine β-synthase (CBS), cystathionine ϒ-lyase (CTH) and 3-Mercaptopyruvate sulfurtransferase (MPST)) in ejaculated and capacitated spermatozoa. In addition, we have quantified and compared these enzymes using Amnis flow cytometry. Our results show that the signal intensity of all enzymes is decreased in capacitated spermatozoa in comparison to ejaculated spermatozoa;

however, only in the case of enzymes, CBS and CTH, was this difference significant (Fig. 11).

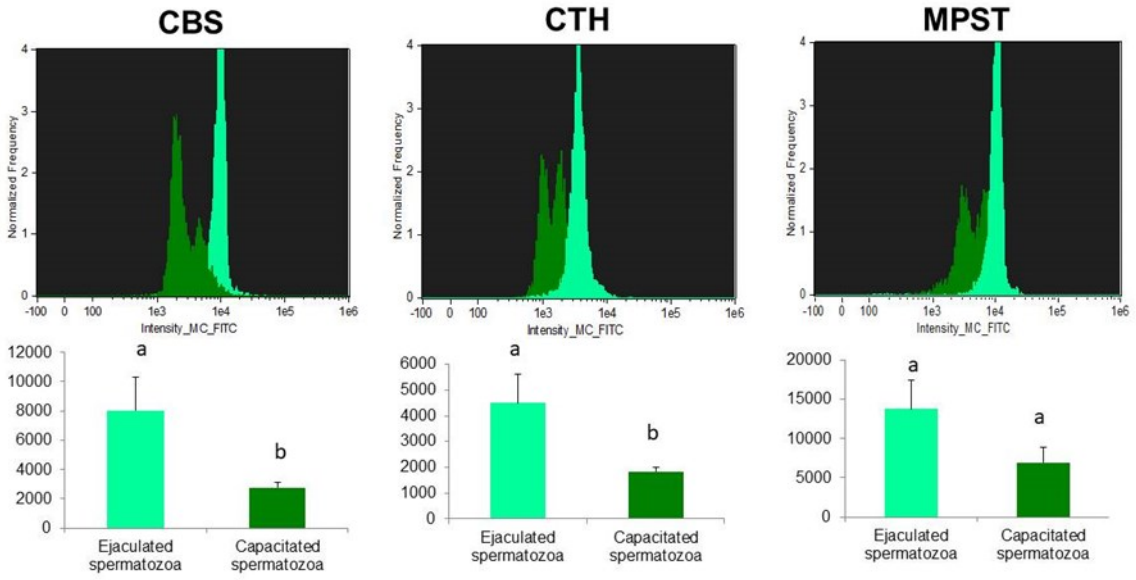

Figure 11. Comparison of H_2S-releasing enzymes in ejaculated and capacitated spermatozoa. Data are expressed as the mean ± SEM. Cystahionine β-synthase (CBS), cystathionine ϒ-lyase (CTH) and 3-Mercaptopyruvate sulfurtransferase (MPST).

H_2S donors preserve sperm head remodeling and Zinc signature

The aim of this analysis was to observe the H_2S effect on sperm viability, acrosome remodelling and zinc signature, as well as select concentration of H_2S donors for subsequent investigation of H_2S on sperm capacitation. The results of the analysis showed that H_2S donors NaHS have a sperm protective effect (Fig. 12), displayed by lower percentage of spermatozoa positive for propidium iodide and disrupt acrosome (PNA). Furthermore, H_2S donors were able to preserve zinc signature 1 as a sign of higher sperm fertilization ability.

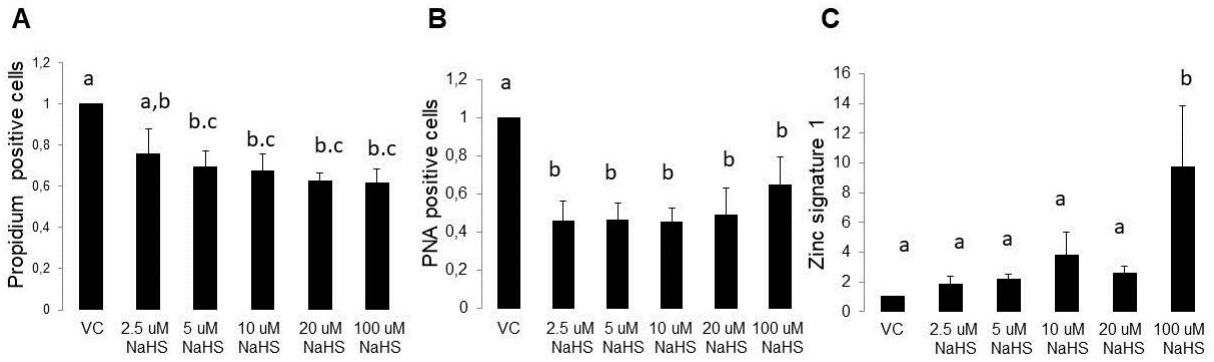

Figure 12. Ejaculated spermatozoa, estimation of NaHS effect on membrane integrity (propidium iodide), acrosomal integrity (PNA), zinc signature. Data are relativized to the control (VC=1) and expressed as the mean ± SEM

H_2S slows down *in vitro* capacitation

The goal of this analysis was to investigate H_2S donors on in vitro capacitation over 4 hours. For this reason, spermatozoa were measured for membrane and acrosomal remodelling, as well as zinc each hour. The obtained results show that capacitation under condition of H_2S slows down (Fig. 13). However, only the concentration 100 uM of NaHS showed significant differences compared to the control group.

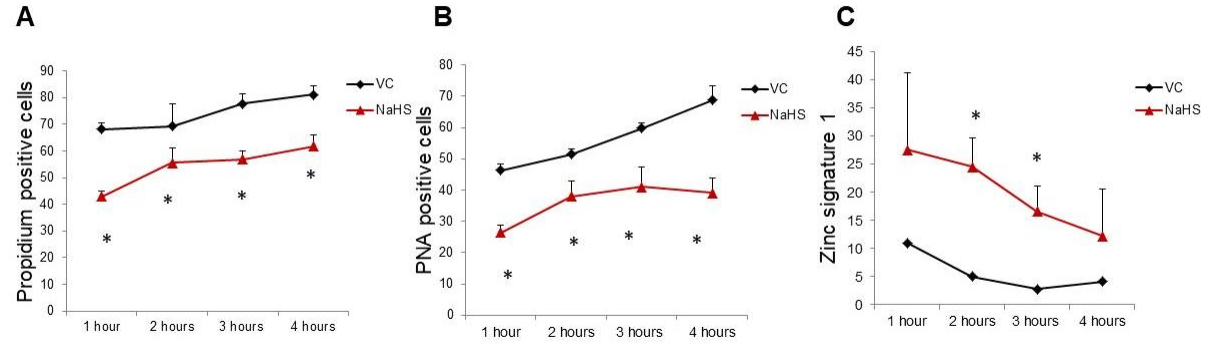

Figure 13. Estimation of membrane integrity (propidium iodide), acrosomal integrity (PNA), zinc signature each hour during *in vitro* capacitation in control and NaHS. Data are expressed as the mean ± SEM.

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

NAD^+-dependent histone deacetylases called sirtuins (SIRT1 – SIRT7) are important posttranslational modifiers of proteins, regulating their physiological role (Bosch-Presegué and Vaquero, 2015). Various epigenetic and non-epigenetic targets were identified(Vaquero *et al.*, 2007; Bosch-Presegué and Vaquero, 2015). Thereby, since, SIRT1 is involved in different pathways such as gene expression, apoptosis, oxidative stress response, senescence and others, role in gametogenesis is not surprising (Mulligan *et al.*, 2011; Das *et al.*, 2014; Ma *et al.*, 2015; Tatone *et al.*, 2015; Yang *et al.*, 2018). The discovery of SIRT1 activators, as well as using SIRT1 knockout, make it possible to improve oocytes maturation and protect gene repression and study the SIRT1 role during protamination and acrosome formation of sperm (Shin *et al.*, 2008; Bell *et al.*, 2014; Li *et al.*, 2015a; Ma *et al.*, 2015; Adamkova *et al.*, 2017; Liu *et al.*, 2017a).

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.

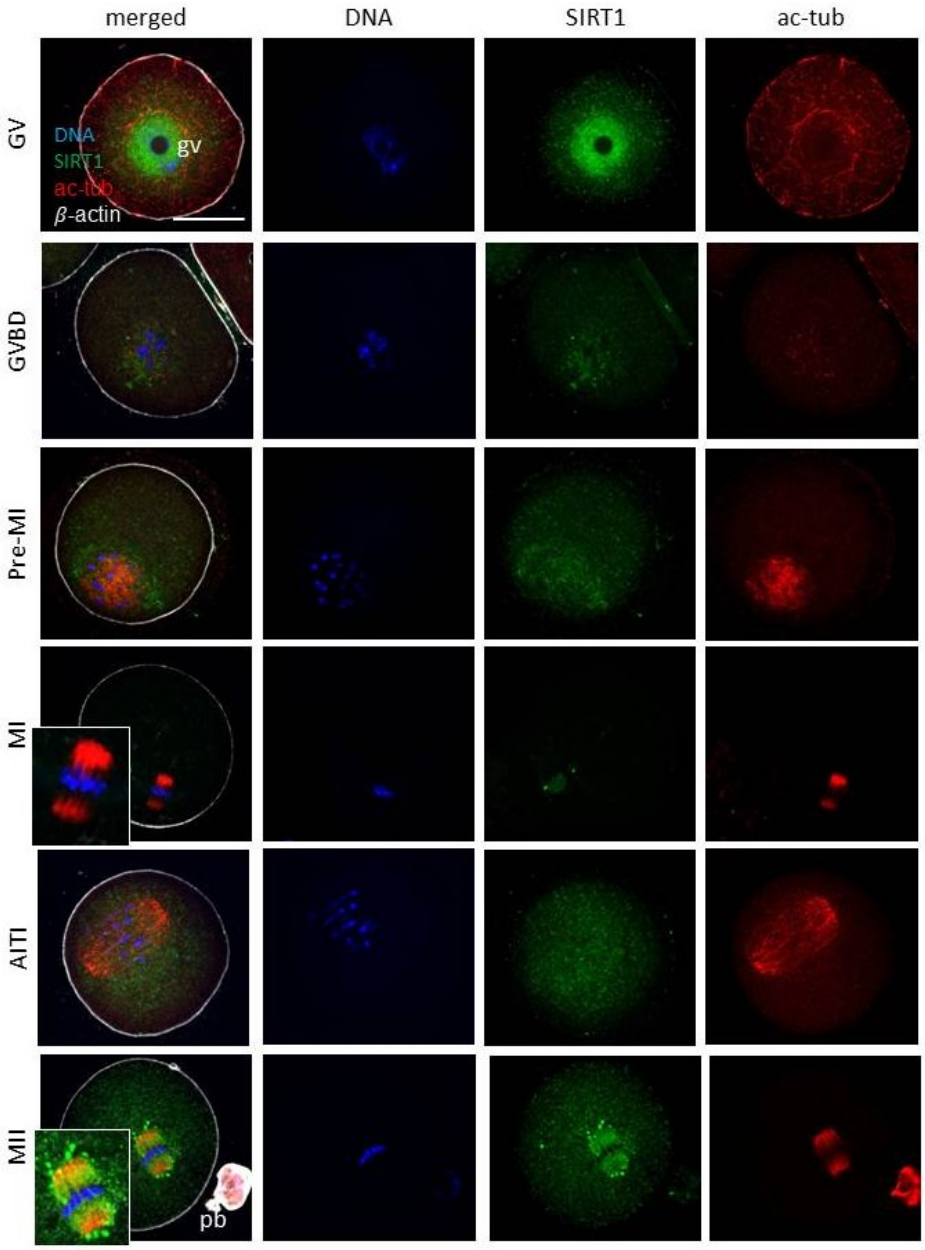

Figure 14. SIRT1 localization in different stages of oocytes maturation. GV, immature germinal vesicle oocyte; GVBD, germinal vesicle breakdown; Pre-MI, pre-metaphase I; MI, metaphase I; AITI, anaphase-telophase transition; MII, metaphase II; gv, germinal vesicle; pb, 1st polar body. The scale bar represents 50 μm.

These considerations have been published in the journal with impact factor factor and is attached as the appendix (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**)

4.3 The environmental impact on fertility through spermatogenesis and oogenesis

During our lifespan, we are exposed to different environmental pollutants, including endocrine disruptors, whose effect is heritable and cumulative not just through life, but also across generations (Knower *et al.*, 2014; Schiffer *et al.*, 2014; Brieño-Enríquez *et al.*, 2015; Vecoli *et al.*, 2016; Bliatka *et al.*, 2017). The most know chemicals with the endocrine disruptive effect are bisphenols, existing in different types (BPA, BPS, BPF, BPE) (Chen *et al.*, 2016; Ullah *et al.*, 2019). The harmful impact of Bisphenol A (BPA) on reproductive health was already observed, and this is reason why it was substituted by its variant bisphenol S (BPS), with the hope that it will be safe and less dangerous (Rosenmai *et al.*, 2014; Björnsdotter *et al.*, 2017; Siracusa *et al.*, 2018). However, as of today, there are more studies describing that BPS in subliminal doses can change the hormonal profile and disrupt gametogenesis (Ivry Del Moral *et al.*, 2016; Ullah *et al.*, 2018; da Silva *et al.*, 2019). Moreover, the effect of BPS on epigenome of female gametes was observed (Žalmanová *et al.*, 2017; Nevoral *et al.*, 2018a) and, therefore, BPS-driven epimutation and its inheritance is suggested.

The ability of endocrine disruptors to interfere with hormones is displayed by the defect during gametogenesis (Cox *et al.*, 2010; Rouiller-Fabre *et al.*, 2014; Ullah *et al.*, 2018). 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 was 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.

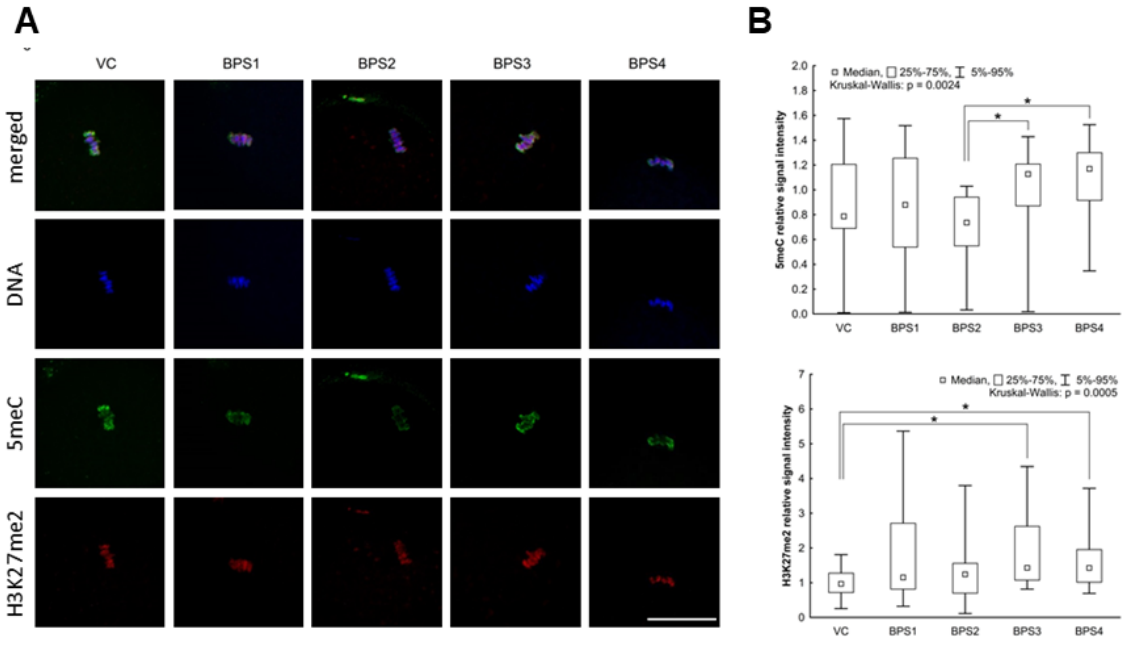

Figure 15. The effect of BPS on epigenetic quality. (A) Immunostaining (B) and relative signal intensity (VC=1) of 5meC and H3K27me2 in metaphase II chromosomes. Scale bar represents 25 μm. Values are expressed as a mean, the boxplot shows 25% and 75% quantiles, as well as 5% and 95% quantiles of observed it in the groups. One star indicates significance at the basic level ($p \leq 0.05$).

In the case of male reproduction, published studies showed that BPS influence the testosterone level and disrupt oxidative stress defence (Ullah *et al.*, 2018). A decrease in sperm concentration and motility were also affected by BPS (Shi *et al.*, 2017). This is in accordance with our study, where we observed the decrease in motility in one BPS concentration (Table 7). 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 (Fig.16). 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 spermatogenesis progression and mitochondria function.

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 epigenetics-based markers.

Table 7. Comparison of mice sperm motility across the groups. VC, vehicle control; BPS1, 0,001 ng.g^{-1} bw. day^{-1}; BPS2, 1 ng.g^{-1} bw. day^{-1}; and BPS3, 100 ng.g^{-1} bw. day^{-1}. Data are expressed as the mean ± SEM. Differing letter superscript in the same row denotes significant differences ($p<0.05$).

	VC	BPS 1	BPS 2	BPS 3	*p* value
Motility (%)	63 ± 19.12 ^{a}	33 ± 16.58 ^{b}	62 ± 10.92 ^{a}	58 ± 12.29 ^{a}	<0.05

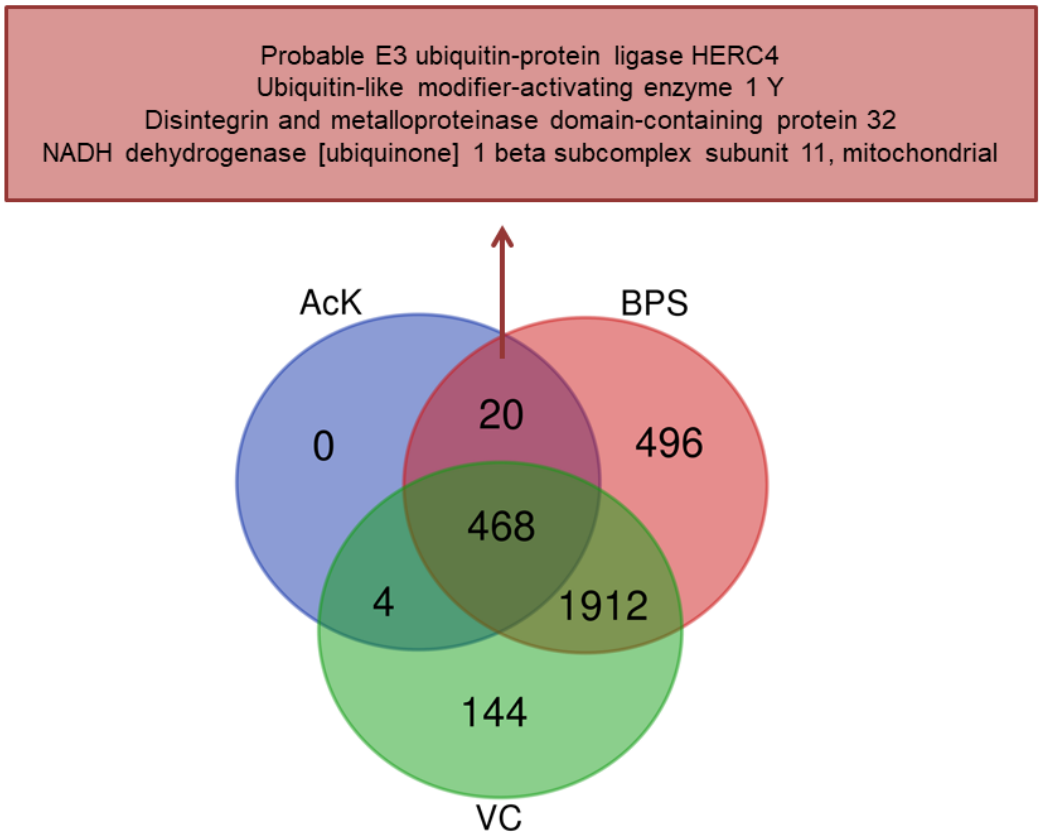

Figure 16. Venn diagram of acetylated proteins. Diagram displays number of acetylated proteins (blue circle, 492 proteins). From them, 20 proteins are acetylated exclusively in the BPS groups (a few of them are emphasized in red box). AcK, total acetylated proteins at lysine residuum; BPS, acetylated proteins found in bisphenol (BPS1-3) groups; VC, acetylated proteins found in vehicle control.

Some results focused on female reproduction have been published in the journal with impact factor factor and is attached as the appendix (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**)

Male reproduction results are prepared for the submission.

Řimnáčová H, **Štiavnická M**, Moravec J, Chemek M, Kolinko Y, García-Álvarez O, Mouton P, Carranza Trejo AM, Fenclová T, Eretová N, Hošek P, Klein P, Králičková M, Nevoral J. Bisphenol S, the old-new hazard for male fertility.

4.4 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 (Tavalaee *et al.*, 2009; Jenkins and Carrell, 2012; Ge *et al.*, 2015; Kutchy *et al.*, 2017; Osman *et al.*, 2018). 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 (Ozanon *et al.*, 2005; Francis *et al.*, 2014; Zhong *et al.*, 2015). In the case of sperm selection, apart from the well-defined markers, use of the non-invasive method is crucial.

In this review, we have offered an outlook on sperm selection methods that may imply epigenetic based markers such as ϒH2AX 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.

Firstly, microfluidic that from all of them is using the most natural principles, like temperature gradient, chemoattractants or the ability of sperm to swim near boundaries (Knowlton *et al.*, 2015; Suarez, 2016). It was shown that sperm selected using microfluidic has lower percentage of DNA damage (Shirota *et al.*, 2016). Accordingly, based on our knowledge about the relation of DNA integrity with incomplete protamination and other associated histone PTMs, it seems that microfluidic is able to take into account epigenome quality.

Secondly, sperm ubiquitin taq assay (SUTI) applying mechanism of ubiquitin based spermatozoa degradation in the epididymis (Ozanon *et al.*, 2005; Baska *et al.*, 2008). Currently there exist several studies providing evidence that ubiquitin is able to recognize spermatozoa with different defects, including DNA fragmentation or incomplete protamination (Ozanon *et al.*, 2005; Varum *et al.*, 2007; Hosseinpour *et al.*, 2014). Furthermore, its efficiency is also proven by the increased fertilization success of spermatozoa selected by SUTI (Ozanon *et al.*, 2005; Eskandari-Shahraki *et al.*, 2013).

The third method that we have described as worthy for epigenetic-based sperm selection is Raman spectrometry (Štiavnická *et al.*, 2017). This method is quite new in reproduction; however, the principle of it to recognize molecules based on their chemical structure and interactions with other molecules offers a huge range for its application. In sperm biology, DNA fragmentation was observed using Raman spectrometry (Huser *et al.*, 2009; Sánchez *et al.*, 2012; Liu *et al.*, 2013). However, studies done on Jurkat cancer cells investigated the application of Raman spectrometry in the evaluation of acetylation status in cells. This creates the promise of its interpolation to sperm biology (Poplineau *et al.*, 2011).

Taken together, spermatozoa selection based on their epigenetic status without damage is possible; however, further investigation is necessary for confirmation of their harmless effect, as well as the estimation of the best method for their clinical application.

These considerations have been published in the journal with impact factor factor and is attached as the appendix (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**).

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 (Jenkins and Carrell, 2011; Vigodner *et al.*, 2013). However, spermatogenesis is quite sensitive to oxidative stress (Wang *et al.*, 2018; Delbes *et al.*, 2010; Kaur *et al.*, 2017; Gunes *et al.*, 2018), and, therefore, we can find a various subpopulation of sperm with different quality in the ejaculate. Some of them are with incomplete chromatin remodelling, and their chromatin is less stable and more sensitive to DNA damage (Castillo *et al.*, 2011; Simon *et al.*, 2011; Dada *et al.*, 2012). Our study showed that spermatozoa with immature chromatin (high DNA stainability, %HDS) are accessible to DNA fragmentation and have generally decreased quality. This observation is in accordance with the studies associating incomplete protamination and decreased fertility (Aoki *et al.*, 2005; Hammoud *et al.*, 2011; Simon *et al.*, 2011; Francis *et al.*, 2014). Moreover, the relation of incomplete protamination assessed by %HDS to assisted reproductive therapy and recurrent pregnancy loss was proven (Bungum *et al.*, 2004; Lin *et al.*, 2008; Evenson, 2013; Jerre *et al.*, 2019). Thereby, successful fertilization is not always related to the quality of embryo development (Schagdarsurengin *et al.*, 2012; Caseiro *et al.*, 2015; Jenkins *et al.*, 2015; Zidi-Jrah *et al.*, 2016; Denomme *et al.*, 2017), mainly if we consider possible epigenetic aberration coming from incomplete protamination, and thus impact on gene expression from paternal line. Accordingly, we have shown that chromatin immaturity is displayed not just by sperm quality, but also by a shift in histone code posttranslational modifications. 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 (Rathke *et al.*, 2007; Goodman *et al.*, 2009; Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010; Wilson *et al.*, 2018), 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.* (2007), we consider H3K4me2 a relevant indicator of spermatozoa quality and chromatin integrity.

The results highlighted the importance of involving epigenetic 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. Moreover, 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 (Mulligan *et al.*, 2011; Tatone *et al.*, 2015; Yu *et al.*, 2017). Accordingly, one of the possible regulators of histone methylation is SIRT1, a member of III class NAD^+-dependent histone deacetylases, called sirtuins (SIRT1-7) (Vaquero *et al.*, 2007; Bosch-Presegué and Vaquero, 2015). Indeed, SIRT1 participates in the regulation of H3K4me2 through histone demethylase lysine-specific histone demethylase 1A (LSD1A). Based on the studies describing improvement of oocytes quality *via* an SIRT1 activator, similar regulation of spermatogeneis would be possible. Although SIRT1 is crucial for sperm development (Coussens *et al.*, 2008), including protamination (Bell *et al.*, 2014), its expression in ejaculated sperm is subliminal without a clearly stated role. However, based on our best knowledge, SIRT1 is required for mitophagy and subsequent embryo development, underlining unique sirtuins' action through non-histone targets (Vaquero *et al.*, 2007; Bell *et al.*, 2014; Bosch-Presegué and Vaquero, 2015; Tatone *et al.*, 2015; Tang, 2016). This is in accordance with our study of SIRT1 during oocytes maturation, where we 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 (Nevoral *et al.* 2019 in press). Since mature spermatozoa have considerably a subliminal amount of SIRT1, with respect to subcellular localization , 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.

Most protein action is dependent on protein posttranslational modifications (PTMs) and their study, in the context of protein description, is needed. Histone deacetylases undergo many PTMs and hydrogen sulphide (H_2S)-derived S-sulfhydration of cysteine residua (Mustafa *et al.*, 2009; Paul and Snyder, 2015) seems to be potent for further study for the following reasons: i) H_2S is the signal molecule, along both cellular and paracrine signalling, with the ability to directly modify the proteins (Munaron *et al.*, 2013), ii) H_2S represent quite a conservative tool for the regulation of protein activity and structure (Vandiver *et al.*, 2013; Zhao *et al.*, 2014), and iii) H_2S is known as neurotoxin; however, it is physiologically released in the cell (Zhang and Bian, 2014).

All these events could be regulated by gasotransmitters (H_2S, NO), gaseous signal molecule that may influence the cell physiology through PTMs of proteins (Nevoral *et al.*, 2016). Considering the involvement of NO in the capacitation through nitrosylation of various proteins, we have looked at the expression, and thus role of H_2S during capacitation (Lefièvre *et al.*, 2007; Yi *et al.*, 2007b). 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.* (2018), 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 (Du *et al.*, 2018). 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 (Cabaton *et al.*, 2011; Brieño-Enríquez *et al.*, 2015; Estill and Krawetz, 2016; Vecoli *et al.*, 2016; Xie *et al.*, 2016). In addition to sperm histone code

and gasotransmission through H_2S, we were focused on selected pollutants for model of environmental-derived modulation of sperm physiological status, including epigenetic factors. Therefore, we have interpolated our knowledge about epigenetic of gametes into the observation endocrine disruptive effect of bisphenol S (BPS), one of most used environmental pollutants (Rosenmai *et al.*, 2014; Adamkova *et al.*, 2017; Nevoral *et al.*, 2018a; Ullah *et al.*, 2019), 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 the 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 highlight 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 underlines 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 markers for the selection of healthier gametes in ART. We expect that the increase application in epigenetics-based methods 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 epigenetic 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 the new epigenome based sperm selection marker H3K4me2 which expression is dependent on spermatozoa quality and chromatin immaturity. This histone modification is obviously regulated by SIRT1, participating in the germ cell maturation. 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 gasotranmitter H_2S, a potent signal molecule modulating protein activity, including SIRT1, has been investigated. H_2S represents a player in sperm physiology, that slow down capacitation possibly through posttranslational modifications (PTMs) of proteins involved in that process. Finally, aforementioned knowledge was utilized for the assessment of endocrine disruptive effect of BPS, which was confirmed on sperm and oocytes through modification of histone code as well as other PTMs of proteins.

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

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8 APPENDIX

Manuscripts

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

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