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Posttranslational modifications of nuclear and nonnuclear proteins in spermatozoa

Ph.D. thesis

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Podpis

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Posttranslational modifications of nuclear and nonnuclear proteins in spermatozoa

Summary

The number of couples who need the help of assisted reproductive technology (ART) has increased over the years. Approximately half of the cases are caused by male infertility, which is often diagnosed as idiopathic infertility. Therefore, the search for male fertility markers will improve male infertility diagnosis, thereby facilitating advanced sperm treatment and selection *via* ART. Posttranslational modifications (PTMs) of sperm nuclear and nonnuclear proteins are suitable candidates for such markers. The PTMs of protamines and histones reflect sperm chromatin maturity and its readiness for fertilization, and accordingly, they can predict the outcome of ART. However, the PTMs of nonnuclear proteins, including cytoplasmic, cytoskeletal, and membrane proteins, reflect the ability of sperm to undergo hyperactivation, capacitation, or acrosome reactions, which are processes essential for fertilization. We hypothesize that the PTMs of nuclear and nonnuclear proteins can reflect sperm quality and, thus, serve as a valuable marker in ART. Additionally, we suggest that the *in vitro* addition of hydrogen sulfide into the sperm-manipulating media improves sperm motility and viability *via* persulfidation. We used Western blot detection in combination with protein identification by mass spectrometry to reach our goals. Furthermore, we used immunocytochemistry and flow cytometry to localize and quantify our targets throughout the whole sperm population. Our results show that the dimethylation of histone H3 at lysine K4 (H3K4me2) is a suitable quality marker of sperm chromatin since an association of H3K4me2 and chromatin condensation of human sperm was found. In addition, we demonstrated that environmental pollutants are able to modulate the well-known PTMs of sperm proteins using mice as an experimental model. This study shows an alteration of the sperm-wide acetylome and phosphorylome in mouse spermatozoa, demonstrating that acetylation and phosphorylation of nonnuclear proteins are suitable candidates for revealing the cause of idiopathic infertility. Finally, we address the presence of hydrogen sulfide and persulfidation, hydrogen sulfide-derived PTM, in male reproduction. In contrast to the aforementioned PTMs, hydrogen sulfide provides a unique possibility of sperm persulfidation *via* exogenous hydrogen sulfide donors, leading to an

improvement of sperm parameters. Altogether, this work demonstrated the importance of PTMs of nuclear and nonnuclear proteins for spermatozoa functionality and suggested their usage as sperm quality markers in ART. Moreover, PTMs of sperm proteins can be modulated during *in vitro* manipulation, and we suggest this technique as a method by which to improve *in vitro* conditions for spermatozoa manipulation.

Key words: (ART), H3K4me2, hydrogen sulfide, PTMs, persulfidation, male infertility

Posttranslační modifikace jaderných a nejaderných proteinů ve spermiích

Souhrn

Kvůli početí potomka vyhledává pomoc asistovaných reprodukčních technologií (ART) stále více párů. Až za polovinou případů párové neplodnosti, stojí mužská neplodnost, která nezřídka končí diagnózou idiopatická (bez známé příčiny). Hledání nových ukazatelů mužské plodnosti, má proto velký význam pro diagnostiku mužské neplodnisti a případně pro selekci spermiích (ne)vhodných pro použití v ART. Post-translační modifikace (PTM) jaderných a nejaderných proteinů se zdají být vhodnými kandidáty pro takové ukazatele. Na jedné straně jsou PTM jaderných proteinů, protamínů a histonů, které odrážejí zralost chromatinu spermie, jeho připravenost na oplození a také kvalitu budoucího embrya. Na druhé straně jsou PTM nejaderných proteinů, zahrnující cytoplasmatické, cytoskeletární a membránové proteiny, které odrážejí schopnost spermie podstoupit hyperaktivaci, kapacitaci a akrosomální reakci, jinými slovy procesy, které jsou nezbytné pro nalezení a oplození oocytu. V této práci předpokládáme, že PTM jaderných a nejaderných proteinů odrážejí kvalitu spermií, a tak mohou sloužit jako selektivní ukazatele pro využití v ART. Dále předpokládáme, že přidání hydrogen sulfanu (H_2S) do *in vitro* manipulačního média, zlepší motilitu a viabilitu spermií, a to prostřednictvím persulfidace. Pro dosažení našich cílů jsme detekovali proteiny pomocí metody Western blotu, v kombinaci s identifikací proteinů hmotnostní spektrometrií. Dále jsme použili metody imunocytochemie a průtokové cytomerie, pro lokalizaci a kvantifikaci našich cílových PTM v populaci spermií. Našli jsme spojitost mezi výskytem di-methylace lysinu K4 nacházejícím se na histonu H3 (H3K4me2) a mírou kondenzace chromatinu spermií, což dělá z H3K4me2 vhodného ukazatele kvality/zralosti chromatinu spermií s možností využití v ART. Další naše experimenty vedly ke zjištění, že látky, běžně znečišťující životní prostředí, mohou ovlivňovat spermie prostřednictvím modulace PTM proteinů. V naší studii jsme pozorovali změny acetylomu a phosphorylomu spermií, které měly souvislost se sníženou motilitou spermií. Nastolili jsme tak důležitou otázkou, zda polutanty z životního prostředí nemohou být příčinou mužské idiopatické neplodnosti. Nakonec jsme se zabývali výskytem sulfanu (H_2S) a PTM od něj odvozená, persulfidace, v mužské reprodukci. Na rozdíl od výše zmíněných PTM,

persulfidace může být u spermií navozena exogenním dodáním donoru H_2S, což může být i jedna z příčin zlepšení parametru spermií po přidání H_2S donoru do media. Tato práce poukazuje na důležitost PTM jaderných a nejaderných proteinů na fungování spermií a navrhuje jejich využití jako ukazatele kvality spermií pro ART. Navíc, PTM proteinů spermií mohou být pozměněny přidáním donoru některé reaktivní molekuly např. H_2S během *in vitro* manipulace, a tak může dojít k výraznému zlepšení přežitelnosti spermií v *in vitro* podmínkách.

Klíčová slova: asistovaná reprodukce, H3K4me2, sirovodík, posttranslační modifikace proteinů, persulfidace, mužská neplodnost

Modificaciones postraduccionales de proteínas nucleares y no nucleares en los espermatozoides

Resumen

En los últimos años, el número de parejas que precisan de biotecnologías reproductivas para concebir hijos ha incrementado exponencialmente. En torno a la mitad de los casos diganosticados son debidos a problemas de infertilidad masculina idiopática. Por lo tanto, encontrar marcadores indicativos de fertilidad masculina ayudarían a predecir el éxito de la aplicación de las diferentes técnicas de reproducción asistida (TRA). Las modificaciones posttraslacionales (MPTs) de proteínas nucleares y no-nucleares del espermatozoide se presentan como perfectos candidatos con los que poder discriminar muestras espermáticas subfértiles. Por un lado, las MPTs de protaminas e histonas son reflejo de madurez de la cromatina espermática y por lo tanto de su capacidad para fecundar el ovocito. Por otro lado, las MTPs de proteínas no nucleares como las citoplasmáticas, estructurales y de membrana, son reflejo de la habilidad espermática para llevar a cabo la capacitación o la reacción acrosómica que preceden la fecundación. Por lo tanto, la hipótesis de esta tesis es que las MTPs de proteínas nucleares y no nucleares pueden ser indicativas de calidad espermática y por lo tanto representar un discriminador eficiente de muestas fértiles para la aplicación de las TRA. Además, proponemos que la adición de ácido sulfhídrico (H_2S) a los medios utilizados para la manipulación *in vitro* de los espermatozoides podría mejorar su funcionalidad *via* la persulfidación de proteínas. Para los diferentes experimentos que forman parte de esta tesis doctoral, se han utilizado técnicas como el Western blot o la Espectometría de masas. Además, la Inmunocitoquímica y la Citometría de flujo nos ha permitido localizar y cuantificar diferentes factores relacionados con la fisiología espermática. Los resultados muestran que, en espermatozoides de humano, la metilación de la histona 3 en la lisina K4 es un prometedor marcador de calidad espermática puesto que está relacionada con la adecuada compactación del ADN. Además, empleando el ratón como modelo experimental, hemos demostrado que la exposición a contaminantes ambientales altera la acetilizacíón y fosforilación de proteínas lo que podría constituir una de las causas de la infertilidad idiopática masculina. Por último, demostramos que la MPT de proteínas mediada por la persulfidación tras la aplicación exógena de H_2S a los espermatozoides

mejora la funcionalidad espermática. En conclusión, esta tesis demuestra que las MPTs de proteínas nucleares y no nucleares del espermatozoide son esenciales para su funcionalidad y demuestra su uso potencial como marcadores de fertilidad masculina en las TRA. Además, proponemos el uso del H_2S como promotor de estas MPTs con el objetivo de mejorar la calidad espermática *in vitro.*

Palabras clave: téncnicas de reproducción asistida (TRA), metilación de la histona 3 en lisina K4 (H3K4me2), ácido sulfhídrico (H_2S), modificaciones posttraslacionales (MPTs) de proteínas, persulfidación, infertilidad masculina.

Content

1. Overview

Currently, 8–12% of couples experience infertility, and almost half of the worldwide cases are caused by male infertility. Over the last 50 years, sperm concentration has declined by 50%, along with an associated increased dependence on assisted reproductive technology (ART)[1,2]. Although the factors contributing to the sperm quality decrease are unclear, there is a reasonable suspicion that environmental pollutants and lifestyle are factors. Therefore, in addition to classic semen parameters (volume, concentration, motility, and morphology), DNA fragmentation or reactive oxygen species (ROS) level detection were included in routine sperm diagnostics. Undoubtedly, these methods have improved sperm diagnostics. However, new sperm quality markers are still required for ART, which many researchers have been working to identify. Posttranslational modifications (PTMs) of sperm histones are suitable candidates for such markers due to dramatic changes in the histone code during spermatogenesis and the inheritance of the epigenetic code incoming with the sperm head during fertilization. In addition to chromatin proteins, nonnuclear proteins are considered in this thesis as crucial targets of PTMs, driving sperm postejaculation fitness, capacitation, and fertilization ability[3–5]. Additionally, sperm histone and nonhistone PTMs can be altered by diet and environmental stress, making protein PTMs a good indicator of environmental pollution[3,4,6]. Accordingly, these findings suggest a high potential of protein PTMs in diagnosing male subfertility and infertility. However, some PTMs remain elusive; therefore, we focused on novel PTMs contribute to redox balance in sperm. Persulfidation (-$(S)_nH$), the hydrogen-sulfide-driven PTM, is obviously a candidate protein modification for the enhancement of sperm assessment, selection, and/or treatment, leading to improved ART outcomes. Nevertheless, further studies are needed to elucidate this PTM and facilitate knowledge transfer to human and/or veterinary reproductive medicine.

2. Aims

The main goal of this thesis was to study the role of PTMs of nuclear and nonnuclear proteins in sperm physiology and their potential use for the improvement of ART *via* the diagnosis of male subfertility and infertility. We hypothesize that PTMs of sperm proteins can respond to environmental conditions and, thus, affect the functionality of key proteins. Special attention was given to histone PTMs carried by spermatozoa, their physiological values, and their potential use as sperm quality markers. Another aspect of the work focuses on nonnuclear PTMs of sperm proteins, with emphasis on poorly described redox PTMs. To achieve this general aim, we propose the following specific objectives related to the three chapters of this thesis:

1. Study of H3K4me2, a histone H3 PTM, as a suitable marker of sperm maturity.

2. Demonstrate how environmental pollutants can alter sperm quality through protein acetylation and/or phosphorylation and, therefore, be an underlying factor in male idiopathic infertility.

3. Describe the persulfidation, a hydrogen sulfide-derived redox PTM, in mammalian spermatozoa, its role in male reproduction and the potential use of hydrogen sulfide supplementation in ART.

The thesis emphasizes the importance of developing new sperm quality markers and approaches, which will improve in vitro conditions for gamete preparation, manipulation, and selection in ART.

3. Literature review

3.1. The path from spermatogonia to oocyte fertilization

A sperm cell undergoes a long journey to achieve the ability to fertilize an oocyte. This journey begins in the testis seminiferous tubules, where diploid spermatogonia give rise to a unique haploid cell, spermatozoon. In addition to chromatin condensation and reduction division, sperm chromatin undergoes dynamic and continuous exchange of canonical histones for histone variants. Histone variants and their posttranslational modifications (PTMs) drive gene expression during spermatogenesis and take control of histone-to-protamine exchange in a process called protamination. Initially, core canonical histones H2A, H2B, H3, and H4 are part of the nucleosome, whereas linker histone H1 binds to linker DNA. These histones are synthesized during DNA replication only, whereas histone variants are replication-independent and can be synthesized at any time of the cell cycle. These variants include ubiquitously expressed histones, including *e.g.,* H2A.X, H2A.Z (belonging to H2A) and centromere protein A and H3.3 (belonging to H3). Interestingly, the testis has the most significant number of specific histone variants, which can be found in the testis only. It is suggested that they play an essential role in genome-wide histone replacement during spermatogenesis and include TH2A, H2AL2 and H2A.B (H2A), TH2B (H2B), H3T and H3.5 (H3) and H1t, H1T2 and H1LS1 (H1)[5,7]. Protamines tightly package the majority of sperm chromatin; however, a small number of residual histones resist the chromatin of mature spermatozoa. Together, nuclear proteins (histones and protamines) and their PTMs are responsible for sperm differentiation, paternal genome preservation, and paternal genome reprogramming during early embryonic development.

After spermatogenesis, sperm chromatin is transcriptionally and translationally silent. Therefore, spermatozoa must rely on already synthesized proteins during sperm maturation in the epididymis and capacitation in the female reproductive tract. Protein PTMs are transcriptionally independent and, therefore, they control and modulate protein activity even in translationally inactive cells. Sperm capacitation (physiological changes that render the sperm able to fertilize) is known to be driven by protein phosphorylation[8]; however, phosphorylation is not the only PTM that participates in this delicate event. Oxidation-reduction (redox) PTMs are candidate regulators which form on sulfhydryl group (-SH) of cysteine, one of the omnipresent and common amino acids. It is well known that reactive

oxygen and nitrogen species promote sperm capacitation. However, the mechanism they use to achieve this is largely unknown, although we suggest that they do so *via* oxidative PTMs. Due to the increase in reactive molecules and oxidative processes during capacitation, it is extremely difficult to resist oxidative stress in spermatozoa. We suggest that sperm-reducing substances such as hydrogen sulfide (H_2S) and persulfidation help maintain redox balance during sperm capacitation. Altogether, the information regarding redox PTMs' involvement in sperm physiology can provide options to improve *in vitro* incubation and manipulation of spermatozoa (cryopreservation, microfluidic chip-based sperm selection, or sperm sorting).

After sperm capacitation, the hyperactivated spermatozoon detaches from the epithelium of the fallopian tube and swims toward the oocyte. Then, the spermatozoon passes through cumulus cells, which, surrounding the oocyte (*corona radiata*), undergoes an acrosome reaction and binds to the *zona pellucida* of the oocyte. As the spermatozoon passes through the *zona pellucida*, it finds itself in the perivitelline space, where it fuses with the oolemma and penetrates the oocyte. Immediately after fertilization, the oocyte completes the 2nd meiosis, and maternal and paternal genomes form pronuclei, which undergo genome-wide reprogramming. PTMs of nuclear proteins are considered fundamental for parental genome reprogramming. In addition to the histone maternal pool recruited during pronuclear formation, residual histones of sperm origin are also essential for pronuclear formation. Moreover, these paternal histones and their PTMs are involved in the expression of genes important in early embryonic development[6,9–11]. It is clear that the sperm histone code affects gene expression during spermatogenesis, but importantly, it controls gene expression during embryogenesis. Therefore, some histone PTMs can reflect sperm quality and readiness for fertilization and simultaneously predict IVF outcomes. Altogether, PTMs of nuclear and nonnuclear proteins have significant potential for sperm diagnosis and improvement of assisted reproductive technology (ART). The following subchapters will be dedicated to each milestone in sperm development with an emphasis on PTMs.

Dynamics of nuclear protein PTMs during spermatogenesis

Spermatogenesis is a complex process that results in highly specialized haploid cells and spermatozoa. Nevertheless, reduction division is not the only dramatic change that sperm chromatin experiences. After meiosis, sperm chromatin undergoes dynamic remodeling, which results in histone-to-protamine exchange. Protamines cause chromatin to become extremely condensed; however, residual histones carry the epigenetic information necessary for spermatogenesis and future embryo development. This chromatin remodeling is controlled mainly by nuclear protein posttranslational modifications (PTMs). PTMs alter the ability of nuclear proteins to bind DNA and DNA accessibility to transcription factors, leading to gene expression changes. The most studied PTMs of nuclear proteins are histone PTMs, including acetylation, methylation, and phosphorylation[7]. In addition to functional groups and histone residues, specific proteins, such as chaperones, transferases, kinases, phosphorylases, histone deacetylases, and demethylases, are essential to establish the histone epigenetic code during spermatogenesis. However, in the following text, these specific proteins will be omitted, focusing only on nuclear protein PTMs and their occurrence and involvement in spermatogenesis.

After a series of mitotic divisions, spermatogonia become the primary spermatocyte and enter the S-phase of the cell cycle, giving rise to tetraploid spermatocytes undergoing meiosis. During the first meiotic division, condensation of chromosomes and induction of double-strand breaks (DSBs) are necessary for homologous chromosome pairing, crossing over and sex chromosome inactivation. DSBs result in phosphorylation of histone H2A. X (γH2A. X), which is a well-known marker of the DNA damage response (DDR) pathway[12]. After genetic recombination, paired chromosomes form a metaphase plate and segregate into the two daughter cells. These cells immediately enter the second meiotic division, resulting in the formation of four haploid round spermatids. Histone 3 (H3) variant centromere protein A (CENP-A) forms pericentromeric heterochromatin, which is crucial for the establishment of centrosomes and necessary for proper chromosome segregation during cell division. Almost all canonical histones are replaced by histone variants and testis-specific histone variants during meiosis and play a crucial role in further spermatid development. These histones determine gene activity during spermatogenesis, orchestrate histone-to-protamine

exchange, and organize the paternal genome for utilization within upcoming fertilization and embryonic development.

Round spermatids are highly transcriptionally active, and therefore, heterochromatin and euchromatin must be strictly established. H3.3 and testis-specific variants H3T and H3.5 and their PTMs are associated with euchromatin[13,14]. In accordance, H3 di- and tri-methylated on lysine residue 4 (H3K4me2 and H3K4me3) is found in genes important for spermatogenesis[11,15,16], while H3K4me2, H3K4me2 and H3K27me3 are enriched in embryonic developmental gene promotors[6,9–11]. Conversely, H3K9me3 is a well-known hallmark of heterochromatin[17,18]. Histone acetylation is generally associated with histone-to-protamine exchange, which is one of the best-described processes along with spermatogenesis. In elongating spermatids, hyperacetylation of H4 on lysine residues K5, K8, and K16 is the first sign of nuclear reshaping. Acetylation opens the chromatin structure that induces histone displacement and incorporation of transition proteins 1 and 2 (TP1 and TP2)[19,20]. This exchange is facilitated by phosphorylation of TPs, which is removed once TP is incorporated into the chromatin. The next step of chromatin reorganization is the replacement of TPs by protamines (PRM1 and PRM2), which is facilitated by the same principles described for histone-TP exchange[21,22]. Other testis-specific histone variants are involved in histone-to-protamine exchange, such as TH2A, H2AL2, TH2B, H1t, H1T2 and H1LS[22]. Alongside the hyperacetylation and testis-specific histone variants, the transient appearance of DSBs by γH2AX helps to exchange nuclear proteins[23]. Thereafter, protamines pack DNA into a highly condensed, stable, inactive toroidal chromatin structure.

Transcriptional activity gradually decreases as histones are eliminated from sperm chromatin during spermatid maturation. Finally, a few histones remain in mature spermatozoa (15% human and 1% mouse), and some studies suggest that they are preferably located in promotors of genes essential for embryonic development. The commonly reported PTMs H3K4me2, H3K4me3 and H3K27me3 have been found in gene regulatory sequences[9,10] and more specifically in imprinted gene clusters, HOX clusters, and developmental transcription factors in the embryo[6]. Other histone PTMs were reported to be passed from spermatozoa to early embryos, H3K9ac in humans or H4K8ac and H4K12ac in mice[24,25]. However, numerous studies suggest the opposite, namely, histone retention over gene-poor regions[26–28] and transmission of genome architecture rather than establishment of the embryonic

transcriptional program. Perhaps these studies[28–30] will bring attention back to protamines, for which PTMs are neglected in sperm-to-embryo transfer studies.

Taken together, any alteration of histone PTMs carried by mature spermatozoa indicates disruption of spermatogenesis. Therefore, histone PTMs can be an excellent marker of male subfertility and infertility. Moreover, some histones of sperm origin are transmitted to the embryo, where altering the "histone code" could lead to fertilization failure or early embryonic loss. Therefore, it is crucial to seek new markers among histone PTMs that could aid in sperm diagnostics and thus improve ART outcomes.

Disulfide bond formation during sperm maturation in the epididymis

After spermatogenesis and adequate morphological changes, immature spermatozoa enter the epididymis. To become mature, they undergo a series of biochemical and functional changes, which lead to the acquisition of motility and fertilization competence. Spermatozoa are transcriptionally and translationally silent; therefore, they must rely on the synthesis of the epididymal epithelium and PTMs of their own proteins. Indeed, phosphorylation, glycosylation, and disulfide bond (-S-S-) formation are the most important PTMs underlying sperm maturation in the epididymis. Phosphorylation occurs predominantly in enzymes and glycosylation in the plasmatic membrane proteins, while -S-S- influence the most important processes that occur during sperm maturation, chromatin condensation, and tail stabilization.

The epididymis is a coiled tubule, which is divided into three different segments: *caput* (closest to the testis), *corpus* (middle part), and *cauda* (connected to the *vas deferens*). Each of these segments exhibits a unique environment and gene expression, which are essential for individual steps of sperm maturation. After entrance into the epididymis, sperm chromatin gradually condenses due to the formation of -S-S-. Disulfide bonds are more abundant in spermatozoa from the epididymal *cauda* than the *caput*[31].

-S-S- results from the oxidation of sulfhydryl groups (-SH) of cysteine, an amino acid frequently found in protamines. This stabilization of sperm chromatin by -S-S- is described across mammals and is thought to protect the paternal genome during its passage through the female reproductive tract toward the oocyte[32,33]. It was found that the ability of -S-S- formation and, thus, chromatin condensation decreases with paternal age[34]. The formation of -S-S- may not be the only alteration of sperm chromatin during epididymal transit. A

recent study showed differences between spermatozoa from the *caput* and *cauda* in H3 and H4 PTMs[35]. These findings challenge the dogma that all chromatin-based epigenetics are established during spermatogenesis. However, further studies are necessary.

The formation of -S-S- also plays an essential role in sperm flagellum, and, indeed, some studies suggest that their formation is necessary for motility competence. Similar to chromatin, flagellum proteins gradually form -S-S- as spermatozoa move from *caput* to *cauda*[32,36–38]. These proteins include the outer dense fiber-1 protein (ODF1), A-kinase anchoring protein 4 (AKAP4), and glycolytic enzymes, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hexokinase-1 and lactate dehydrogenase. Together, these proteins form a scaffold that surrounds and supports the flagellum and simultaneously supplies it with ATP needed for sperm motility. Fatty acid-binding protein 9 (FABP9), voltage-dependent anion channel 2 (VDAC2), and *zona pellucida* binding protein (ZPBP) were also reported to undergo -S-S- formation[36–38]. -S-S- probably stabilize these proteins and render them resistant to premature capacitation and chromatin decondensation in the female tract.

Redox-driven PTMs of sperm proteins during capacitation

Although spermatozoa show progressive motility immediately after ejaculation, they cannot fertilize the oocyte. First, spermatozoa have to reside for several hours in the female reproductive tract and undergo a series of biochemical changes, collectively named capacitation. Capacitation results in acrosome preparation for exocytosis, remodulation of the plasmatic membrane, and hyperactivated motility, events necessary for oocyte fertilization. It has been known for many years that capacitation is derived from reduction-oxidation (redox) events caused by the generation of reactive oxygen, nitrogen and sulfur species (RONSS). However, it is still not completely understood how RONSS are generated and which mechanisms they use to orchestrate whole capacitation. One possible mechanism of redox signal transduction is *via* redox-derived PTMs of the -SH of sperm proteins. Unfortunately, recent knowledge of redox PTMs is limited to the -SH/-S-S- content and neglects the existence of other redox PTMs within the capacitation. Research on redox events that drive capacitation is severely lacking, although new findings could improve several ART approaches, such as cryopreservation, gamete cultivation, or *in vitro* fertilization (IVF).

Once a spermatozoon enters the female reproductive tract, its intracellular environment changes rapidly. The influx of ions, especially HCO_3^-, increases intracellular pH and 3′,5′-cyclic adenosine monophosphate (cAMP) and, thus, triggers the cAMP-dependent protein kinase A (PKA) activation cascade. PKA then activates many proteins, including tyrosine kinases, leading to a global increase in tyrosine phosphorylation. Therefore, phosphorylation is the most widely used marker of sperm capacitation[8,39]. However, acetylation of sperm proteins increases during capacitation as well, suggesting its involvement in capacitation[40]. These processes are accompanied by the constant removal of cholesterol from the plasma membrane, which changes its fluidity and permeability for ions. As capacitation progresses, intracellular Ca^{2+} increases, the plasmatic membrane hyperpolarizes, and the actin cytoskeleton reshapes because of sperm preparation for hyperactivation and acrosome exocytosis[41].

RONSS intervenes in all the processes mentioned above and influences capacitation from beginning to completion. The most widely studied reactive molecules include superoxide anion (O_2•), hydrogen peroxide (H_2O_2), nitric oxide (NO•), and peroxynitrite (ONOO•). However, the sources of these molecules are still being discussed. Some of them probably arise as a byproduct of metabolism (O_2•), and other RONSS are produced enzymatically (*e.g.,* NO by nitric oxide synthase)[42–44]. In general, RONSS promotes cAMP synthesis accompanied by the activation of PKA and tyrosine phosphorylation[45]. Moreover, they inhibit tyrosine phosphatase activity, thereby accelerating phosphorylation[46,47]. RONSS also oxidizes cholesterol and, thus, facilitates its removal from the plasma membrane[48]. Finally, they stimulate a self-perpetuating production of RONSS[42]. Interestingly, many enzymes involved in capacitation contain cysteine, whose sulfhydryl group (-SH) renders them susceptible to RONSS modulation[49]. Cysteine then activates and deactivates proteins, depending on which redox PTMs arise on it. Most redox PTMs are reversible, continuously replacing each other, making them a unique pathway of RONSS signaling transmission. Although there is a wide range of redox PTMs derived from the oxidative state of cysteine sulfur, some of them are considered essential: disulfide bonds (-S-S-), nitrosylation (-SNO), and sulfenylation (-SOH) belonging to oxidating PTMs, and glutathionylation (-SSG) and persulfidation (-$(S)_n$H) belonging to reducing PTMs[50].

The formation of -S-S-, one of the most studied redox PTMs, is well described during sperm maturation in the epididymis and capacitation. While sperm gain -S-S- as they pass through

the epididymis, they lose them when capacitated, and more free -SH are detected[51,52]. Moreover, loss of -S-S- from the sperm surface promotes the release of capacitated spermatozoa from the sperm reservoir in the fallopian tube[53]. This massive -S-S- reduction indicates the presence of a reducing agent during sperm capacitation. Such an agent could be glutathione or hydrogen sulfide (H_2S) (causing -SSG and -$(S)_nH$, respectively), both found in spermatozoa and the female reproductive tract[54–56]. Unfortunately, the involvement of these molecules in the capacitance process has not been clarified. Additionally, free -SH resulting from -S-S- breakdown are available for other redox PTMs, thus providing more opportunities for protein regulation and supporting the idea of a complex molecular apparatus of sperm redox control[42,49].

Another well-known reactive molecule promoting sperm capacitation is NO. This molecule causes -SNO of proteins, which are important for sperm physiology, including ryanodine receptors, the Ca^{2+} channel, Tubulin, Glutathione-S-transferase, Heat shock proteins, AKAP, Voltage-dependent anion-selective channel protein, and Semenogelin[57]. Some of these proteins are not directly connected with capacitation, but they contribute to it indirectly *via* participation in cell metabolism, maintaining redox balance, and/or stabilizing the flagellar cytoskeleton. -SOH is another reversible redox PTM that modifies Actin, Peroxiredoxin, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[58]. Under oxidative conditions, -SOH is further oxidized to sulfinic (-SO_2H) and sulfonic (-SO_3H) acids, which are irreversible redox PTMs and cause protein damage. This deleterious hyperoxidation can be avoided if -SOH reacts with a neighboring -SH to form -S-S-. Alternatively, reducing agents, such as glutathione and H_2S, can reduce -SOH and form -SSG or -$(S)_nH$, respectively[59]. Indeed, it has been shown that -$(S)_nH$ has the same protein targets as -SNO or -SOH[50,60]. Moreover, some studies suggest that -SNO and -SOH operate as precursors for -$(S)_nH$ formation. This was supported by the identification of persulfidated proteins in human spermatozoa, including AKAP, GAPDH, tubulin, and semenogelin[61].

Based on previously published studies, low levels of reactive oxygen and nitrogen species, causing oxidative PTMs, are undeniably essential for capacitation, but their overproduction leads to oxidative stress, cell damage, and disruption of capacitation[44]. Therefore, reducing PTMs, such as glutathionylation and persulfidation, is desirable for slowing oxidation and, thus, preventing oxidative stress development. Altogether, redox PTMs create a very sophisticated signaling system that regulates protein activity and simultaneously contributes

to redox balance, which is vital for sperm capacitation. Not much is known regarding redox PTMs, and an improvement in this understanding could yield improvement of sperm incubation under *in vitro* conditions, cryopreservation, and preparation of insemination dosage.

Fertilization and maternal-to-zygotic transition

After fertilization, maternal and paternal genomes are transcriptionally silent, and, therefore, the emerging zygote has to rely on oocyte-stored RNAs and proteins at the beginning of development. First, both parental genomes must be epigenetically reprogrammed, including genome-wide demethylation, protamine-to-histone exchange, histone PTM changes and pronuclear development. This reprogramming is the first step toward maternal-to-zygotic transition (MZT), resulting in totipotent zygotes[62]. The formation of paternal and maternal pronuclei (PN) before their syngamy can be divided into 6 morphologically different stages, PN0 – PN5 **(Fig. 1)**[63]. After the first S-phase and pronuclei syngamy, which is followed by one or several rounds of mitotic divisions, the embryo genome is activated (EGA) in a species-specific manner. Shortly afterward, the embryo loses its potency within each subsequent cleavage, resulting in the first cell fate decisions. During this process, gene expression is determined by *de novo* DNA methylation, leading to cell differentiation and organogenesis[62]. It is undisputed that sperm-born epigenetic markers, especially nuclear protein PTMs, play an essential role in EGA, where histone PTMs are responsible for expressing, *e.g.*, the HOX gene cluster, microRNA cluster and transcription and signaling factors[6].

As the spermatozoon penetrates the oocyte, the oocyte completes the second meiosis and extrudes the second polar body into the perivitelline space. At the same time, the paternal genome undergoes chromatin remodeling, including protamine-to-histone exchange, which is accompanied by various changes in PTMs of protamines and histones. Disulfide bonds between protamines are reduced[64], and protamines are phosphorylated[65], resulting in a gradual decondensation of paternal chromatin. Subsequently, maternally-origin H3.3 and H4 are recruited and incorporated into the paternal genome. Therefore, asymmetry between parental pronuclei can be observed when H3.3 is predominantly found in the paternal pronucleus at the beginning of development (approximately PN0 – early PN3)[66]. Histones H2A and H2B are also part of emerging nucleosomes, but their synthesis is dependent on

replication, and therefore, they are incorporated into the nucleosomes probably during S-phase. Nevertheless, the timing of H2A and H2B incorporation into the paternal genome remains largely unclear[67]. Other asymmetries between pronuclei can be observed in histone acetylation, characterizing the paternal pronucleus, and repressive markers H3K9me2, H3K9me3, and H3K27me3, representing the maternal pronucleus[68].

DNA demethylation is another essential event in parental genomic reprogramming and occurs asymmetrically in both pronuclei. Whereas demethylation is an active process in the paternal pronucleus and occurs shortly after fertilization (PN1 – PN3), the maternal pronucleus remains untouched until the first mitotic division. DNA methylation along with repressive histone markers keeps the maternal genome transcriptionally inactive, while the paternal genome is partially activated in the process called the minor wave of EGA (PN3-PN4 in mice, two-cell stage embryo in humans). After the syngamy of parental pronuclei, the zygote enters mitotic cell division. The maternal genome is gradually demethylated and starts to be transcriptionally active within the cell divisions. This process is called the major wave of EGA, which occurs in two- and eight-cell-stage embryos in mice and humans, respectively. When the embryo passes through minor and major waves of the EGA, the MZT can be considered complete, and the embryo is capable of continuing development by its own transcriptome[67].

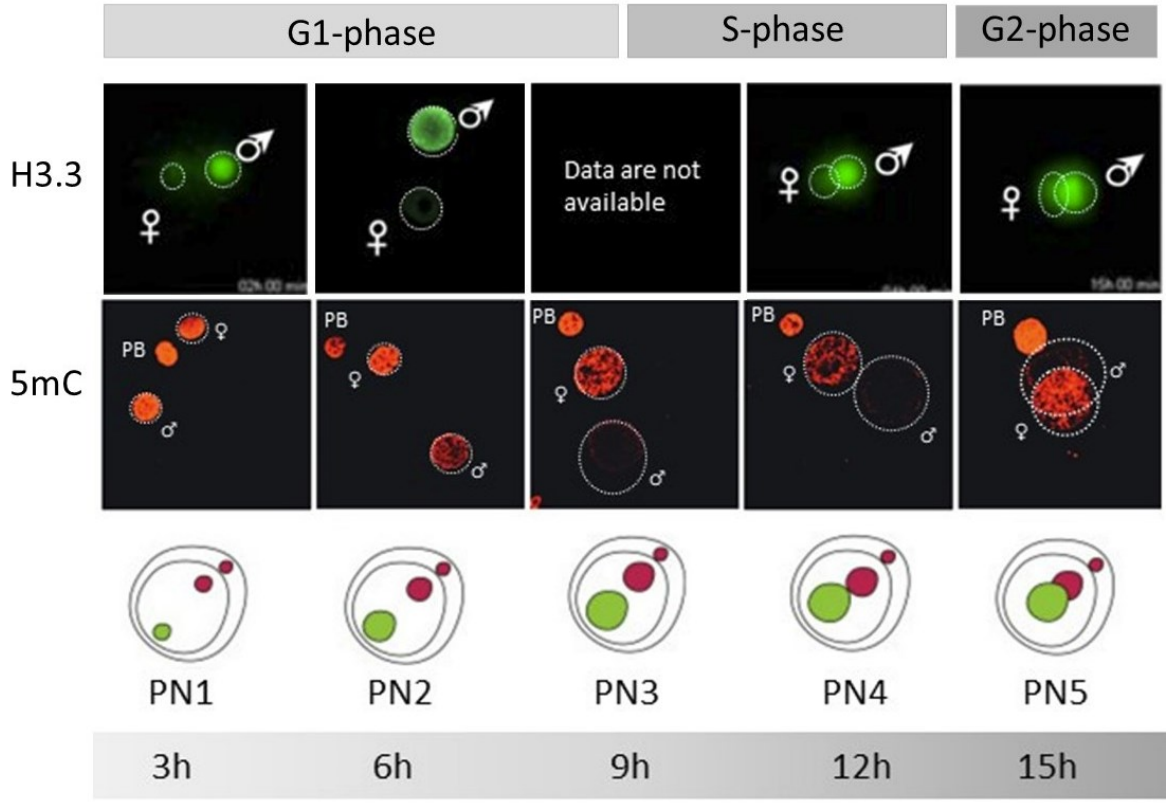


Figure 1. Development of parental pronuclei after fertilization, with an emphasis on histone variant H3.3 incorporation and paternal genome demethylation over time, cell cycle and pronuclear stages (PN1 – PN5). H3.3 is incorporated predominantly into the paternal genome immediately after fertilization. This asymmetry in H3.3 lasted until PN3, after which time H3.3 was incorporated into the maternal genome. Demethylation (loss of the 5-methylcytosine signal) of the paternal genome is the most prominent event in the zygote, and it occurs exclusively in the paternal pronucleus. Demethylation of the paternal genome is complete in PN3.

The paternal pronucleus is displayed in green, and the maternal pronucleus is displayed in red in schematic images PN1 – PN5. The given time corresponds to the time after fertilization. The image has been modified from previously published data[66,69–72].

Role of PTMs of sperm nuclear protein in maternal-to-zygotic transition

In addition to paternal genetic information, spermatozoa carry epigenetic information preserved in noncoding RNAs, DNA methylation, and nuclear protein PTMs. Most of these epigenetic markers are erased shortly after fertilization during epigenetic reprogramming. However, some of them persist to the blastocyst and are necessary for embryonic development. Sperm-born PTMs of nuclear proteins, predominantly histones, can be divided into PTMs that facilitate protamine to histone exchange, participate in cell division, and control embryonic gene expression. Although sperm-driven histones have been intensively studied, their role in embryonic development is still far from being understood.

As mentioned in the previous subchapter, protamines from the paternal genome are rapidly replaced by oocyte histones immediately after fertilization. This process is facilitated not merely by disulfide-bound disruption and protamine phosphorylation; hyperacetylation of H4 on K8 and K12 of sperm origin also contributes to this protamine-histone exchange[73]. Other studies suggest histone retention in noncoding areas, such as pericentromeric, centromeric, and telomeric regions. These regions are essential for embryo genome architecture and mitotic division; therefore, histone variants, creating these specific chromatins, are crucial. The aforementioned histones, including the testis-specific H2A variant H2AL1/L2, creating pericentromeric heterochromatin[29], the H3 variant CENP-A, which is part of centromeric heterochromatin[74], and the testis-specific H2B variant TH2B, were also reported in telomers[75]. In addition, histone PTMs have recently been abundantly investigated in embryonic developmental gene expression. Among all sperm-born histones, studies are most often focused on H3, specifically on H3K4me2, H3K4me3, and H3K27me3. H3K4me2 was found in promotors of genes related to developmental transcription factors[16]. H3K4me3 was found in HOX genes, noncoding RNA clusters, paternally expressed imprinted loci, and genes important in pluripotency (*Klf5* and *Sox7/9*)[6]. Sperm-born H3K27me3 is often associated with the polycomb repressive complex, which inactivates targeted genes[76]. Therefore, H3K27me3 is enriched in HOX and other developmental genes, inactivated in the preimplantation embryo, but awakened later during embryonic development[6,9,10]. In contrast, many studies suggest sperm-born-histone retention predominantly in repetitive DNA elements, involving transposon short and long interspersed nuclear elements (SINEs and LINEs, respectively)[27,28,77]. Although these elements represent

noncoding regions, they are considered to be crucial regulators of early embryonic development. More specifically, Lambrot et al. (2021)[76] focused on H3K4me3 and reported that it is preferentially distributed throughout SINEs and other repeat elements involved in embryonic development[78].

The histone code, which is established during spermatogenesis and carried by the mature spermatozoon, has become an important subject related to epigenetic inheritance. Indeed, Lismer et al. (2020)[3] and Siklenka et al. (2015)[79] showed that disruption of H3K4me2 and H3K4me3 during spermatogenesis leads to improper methylation of H3K4 in the embryo, giving rise to offspring with various developmental defects. Furthermore, they demonstrated that this epigenetic alteration carried by spermatozoa is transgenerationally transmitted[79,80].

Altogether, these results demonstrate that sperm nuclear proteins brought to the oocyte have many different functions. While some of them serve as a template for paternal genome reprogramming, others aid in chromosome formation, and some are essential for embryonic gene expression. There is no doubt that sperm PTMs are essential molecular signs of sperm fertilization and developmental competence.

3.2. PTMs of sperm proteins: impact and medical perspectives

There are an increasing number of couples in Western countries who cannot conceive naturally and need the help of reproductive clinics[81,82]. This unfavorable trend is often caused by postponed parenthood and advanced parental age, lifestyle, and the increasing pressure of environmental pollutants. In general, advanced maternal age and female infertility are emphasized as important factors in this trend, but male and female infertility are equally involved in conception failure. Moreover, unlike oocytes, spermatozoa are constantly renewed throughout a man's life. Therefore, it is more than logical to be focused on sperm-based prevention, diagnosis and treatment of couple infertility, as well as sperm selection for ART. Based on our studies, I suggest that many markers reflecting sperm quality can be found among PTMs. For example, histone PTMs carried by spermatozoa reflect sperm readiness for fertilization and define embryo quality[3,79,80,83]. The need to track these modifications is highlighted due to their plasticity, while they can be altered throughout the father's life *via* diet, lifestyle, diseases, treatments, and/or environmental pollutants[3,84]. There is a reasonable suspicion that PTMs can be disturbed through environmental factors,

not only in histones but also in other proteins important for the proper function of spermatozoa, as indicated in our previous study[85]. Altogether, these results demonstrate that PTMs as a diagnostic tool can provide helpful information on semen quality and its fertilization ability. Moreover, a better understanding of redox PTMs' involvement in sperm physiology could guide us to maintain redox balance and keep oxidative stress under control during sperm manipulation under *in vitro* conditions (cryopreservation, sperm sorting, and microfluidic chip-based sperm selection).

Improvement of sperm selection to increase ART efficiency

Sperm quality estimated from sperm concentration, motility, and morphology appears to be insufficient for current ART. Therefore, additional analyses of DNA fragmentation[86,87], ROS levels[88], and the presence of anti-sperm antibodies in ejaculates are often used in sperm diagnosis. Interestingly, these advanced analyses do not usually correlate with the results of traditional methods; therefore, advanced methods are becoming a powerful tool for treating couple infertility. Despite numerous advanced analyses, there is still a large demand for innovative sperm selection methods based on thermotaxis[89] or rheotaxis[90] and new sperm quality markers, which will help to improve ART. Histone PTMs appear to be suitable candidates. Histone PTMs could reflect impaired spermatogenesis, including incomplete protamination, inconsistencies in histone retention[83], and/or incorrect methylation of residual histones[79].

Indeed, some aberration of H3K4me and H3K27me occurrence in developmental genes was found in spermatozoa of infertile men[90]. However, spermatogenesis disruptions should not directly affect the fertility of the spermatozoon but can affect embryo development. For example, alteration of H3K4me3 in spermatozoa leads to various developmental defects in offspring[3,79,80]. Moreover, Lismer et al.[3] suggested that H3K4me3 in spermatozoa and, subsequently, in the embryo is altered by the father's diet[3]. Unfortunately, not only diet but also environmental pollutants could perturb the sperm histone code and, thus, the health of the embryo. For example, bisphenol A (BPA), a well-known endocrine disruptor and ubiquitous environmental pollutant, increases the occurrence of H3K9me3 in fish spermatozoa[4]. Similarly, histone H3 was hyperacetylated (H3K9Ac and H3K27Ac) in spermatozoa and embryos from exposed fish males[91]. Obviously, PTMs of sperm histones are receptive to the environment and lifestyle; subsequently, they seem to be good candidates

for sperm quality markers, which especially reflect the quality of the embryo. Unfortunately, only a few recent studies have focused on this topic. Therefore, histone PTMs will not likely become routinely used markers in ART for a while.

Equally good markers for determinizing sperm quality could be PTMs of nonnuclear proteins, including enzymes, membrane proteins, and cytoskeletal proteins. After spermatogenesis, spermatozoa are transcriptionally and translationally inactive, and most of the events are directed by PTMs of proteins. Moreover, after the spermatozoon loses almost all its cytoplasm, its ability to resist toxicants and oxidative stress is significantly limited. Based on recent knowledge, we suggest that PTMs monitoring either the whole proteome or individual proteins can serve as a suitable marker for the diagnosis, selection, and treatment of spermatozoa.

Our study demonstrated that the acetylation and phosphorylation of the whole sperm proteome (acetylome and phosphorylome) of males exposed to pollutants differed from the acetylome and phosphorylome of the control group[92]. In practice, this would mean that anamnesis together with PTM markers would lead to the diagnosis of sub/infertility and its treatment (at least the temporary restriction of certain compounds in the environment). PTMs of sperm proteins reflect lifestyle, diet, and the individual's environment, and they could assist in identifying the causes of infertility in men diagnosed with idiopathic infertility. For unknown reasons, the theme of PTMs as an indicator of male sub/infertility is not well studied, even though it has great potential in subfertility and infertility diagnosis and treatment.

Proper choice of sperm PTMs for use in ART

Progress in ART has revolutionized many fields. It has been used to accelerate animal breeding[93], represents tools for saving endangered species[94], demonstrates the possibility for utilizing engineering techniques in human therapies (*e.g.,* therapeutic cloning)[95] and has been used for infertility treatment[96]. It is evident that ART is indispensable in the modern era. A common feature of all ARTs is the *in vitro* manipulation of gametes, which is accompanied by negative effects on sperm, such as oxidative damage[97], a decrease in sperm motility and vitality, premature capacitation[98] and acrosome reactions[99], and epigenetic alterations[100]. These concomitant events impair ART outcomes and worsen its success rates. Indeed, offspring conceived by ART more often suffer from low birth weight, preterm birth, and birth defects, which are primarily issues in humans[100,101]. Generally, the improvement of *in vitro* manipulating media and procedures is necessary to prevent these negative events.

Research on physiological processes, such as sperm capacitation, acrosomal reactions, and fertilization, has great potential to improve the *in vitro* manipulation of gametes. Accordingly, sperm capacitation is controlled by reactive species, which slightly shift the sperm redox balance in favor of oxidative processes during capacitation[44]. Therefore, oxidative stress and sperm damage can finally occur instead of capacitation under *in vitro* conditions. This phenomenon can be prevented by i) avoiding oxidative stress or ii) supplying media with antioxidants. Hydrogen sulfide (H_2S) is an interesting molecule belonging to the gasotransmitter family, with a cytoprotective effect on cells, including spermatozoa. Accordingly, supplementation with H_2S improved sperm vitality and motility under oxidative stress-causing conditions[102]. Moreover, Wang et al.[103] found that asthenozoospermic patients have less H_2S in seminal plasma than normozoospermic patients. In addition, H_2S supplementation led to sperm motility improvement in these asthenozoospermic patients[103]. The therapeutic effect of H_2S on cell survival has been discussed in many scientific journals; however, the mechanism of H_2S action has to be elucidated to provide efficient H_2S-based treatment. A revolution in this topic was brought forth by our previous study[61], where we detected all H_2S-releasing enzymes in spermatozoa and described persulfidation (H_2S-drive PTM) of sperm protein for the first time. Persulfidation is one of the redox PTMs, which are described in detail in the chapter "Redox-driven PTMs of sperm proteins during capacitation". Importantly, persulfidation prevents

protein oxidative damage and, together with other redox PTMs, represents an essential signaling pathway of protein regulation. These facts provide strong evidence that persulfidation is a mechanism of H_2S action.

Although the study of the therapeutic effects of H_2S on sperm is still in its initial phases, it can be suggested that i) H_2S could improve the spermatozoa of asthenozoospermic patients with H_2S deficiency and, thus, facilitate the performance of less invasive ART, such as insemination, over more invasive approaches; ii) H_2S could help to defend spermatozoa against oxidative stress; and iii) H_2S can improve the timing of sperm capacitation. Although supplementation of H_2S by its donors is simple, better knowledge on effective and nondeleterious H_2S doses is still need to be established. Assessment via advanced parameters, such as the abundance of free thiols, level of redox PTMs, and fertilization ability of sperm, can help to identify the appropriate therapeutic dose.

4. Overview of our results

4.1. H3K4me2 as a novel epigenetic marker for sperm quality assessment

Proper chromatin remodeling during sperm development is necessary for delivery of the intact paternal genome to the oocyte and, thus, successful fertilization. Epigenetic markers play essential roles in chromatin remodeling, including PTMs of residual histones. These histones are believed i) to be in the promoters of genes essential for early embryonic development, ii) to be in regulatory sequences such as transposons and enhancers and/or iii) to bring information about the genome architecture into the embryo. Histones H3 and H4 and their variants are frequently located in genes essential for spermatogenesis. In addition, H3, H4, and their variants are most often transferred histones from spermatozoa to the embryo; therefore, their PTMs attract the most attention. Dimethylation of histone H3 at lysine K4 (H3K4me2) is a euchromatin-associated modification located in genes essential for spermatogenesis. Moreover, it is known to be transmitted to the embryo within the sperm histone code. Based on our best knowledge, we consider H3K4me2 to be a crucial sperm factor; therefore, we have chosen it as a suitable candidate for indicating sperm quality. For the H3K4me2 evaluation, we chose immunodetection in combination with flow cytometry of human semen samples of various qualities: normozoospermic (N), asthenozoospermic (A), and oligoasthenozoospermic (OA). We investigated the impact of H3K4me2 content on conventional sperm parameters (volume, concentration, and motility) and less conventional parameters, such as DNA fragmentation and DNA stainability.

Methods

Semen samples

The study was approved by the Ethics Committee of Charles University, Faculty of Medicine, in Pilsen (238/2016). A total of 99 semen samples were assessed, 22, 63 and 14 of which were N, A, and OA, respectively. Spermatozoa were washed from seminal plasma and kept in culture medium (Biggers-Whitten Whittingham medium) in an incubator (37 °C, 5% CO_2).

Sperm chromatin structure assay (SCSA)

The SCSA assay detects DNA breaks and abnormalities in chromatin packaging *via* flow cytometry of acridine orange (AO)-stained spermatozoa. In this study, SCSA was performed according to Evenson and Jost[104]. Briefly, aliquots of semen samples were frozen in liquid nitrogen and stored at -80 °C. On the day of the assessment, thawed samples were treated for 30 s with low-pH buffer to open the DNA strands at the break sites and stained with AO. The AO was excited with a 488-nm laser; a 700/54BP filter detected red fluorescence, and a 537/32BP filter detected green fluorescence. The red emission represents DNA breaks, expressed by the DNA fragmentation index (%DFI). The green emission represents the degree of chromatin packaging, also referred to as chromatin immatureness, expressed by high DNA stainability (%HDS). Data were collected from 5000 cells and were analyzed by WEASEL Ver. 3.

H3K4me2 detection by flow cytometry

H3K4me2 detection by flow cytometry was performed according to a previous study[105]. Briefly, spermatozoa were fixed in 4% paraformaldehyde and permeabilized (0.5% Triton X-100 in PBS) for 15 min. Each specimen was divided into a stained sample and a negative control. Stained samples were incubated with polyclonal rabbit anti-H3K4me2 antibody (1:100) for 60 min. Subsequently, stained samples and negative controls were incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:200) for 30 min. A 488-nm laser was used for fluorescence excitation, and emission was detected by a 537/32BP filter. The resulting H3K4me2 fluorescence intensity was obtained after subtraction of the fluorescence intensity of the negative control from the fluorescence intensity of the stained sample. Data were collected from 5000 cells and analyzed by WEASEL Ver. 3.

Western blot analysis of H3K4me2

Based on the SCSA results, samples were divided into %HDS ≤ 15 and %HDS > 15 groups according to Evenson[87], and their H3K4me2 levels were compared by Western blotting. For this purpose, we pooled samples from five patients and loaded approximately 10 million spermatozoa per well.

Samples were washed with PBS, dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 120 mM DTT, 40 mM TRIS base) and incubated for 30 min on ice. The prepared lysates were then solubilized in Laemmli buffer (Bio–Rad, France) and boiled for 5 min at

97 °C. Thereafter, samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12.5% separating gels and blotted using the Trans-Blot Turbo Transfer System onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in 1% BSA in TBS with 0.05% Tween-20 (TBS-T) for 60 min at room temperature. H3K4me2 was incubated with rabbit polyclonal anti-H3K4me2 (1:1,000; Abcam, UK) overnight at 4 °C. A mouse polyclonal antibody against α tubulin was used as a loading control (1:1,000; Sigma–Aldrich, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG secondary antibodies (1:15,000; Thermo Fisher, USA) were applied for 60 min at room temperature. Target proteins were visualized by ECL Select Western blotting Detection Reagent and the ChemiDoc MP System (Bio–Rad, France). Densitometry analysis was performed by Image Lab 6.0.1 software (Bio–Rad, France).

Statistical analysis

Data were analyzed using statistic software (Statistica or GraphPad Prism 8). We used parametric (ANOVA) or nonparametric (Friedman test) tests based on Shapiro-Wilks normality distribution tests. P values ≤ 0.05, .01, .001, and .0001 were considered statistically significant and are indicated with asterisks (*), (**), (***), and (****), respectively.

Results and Discussion

We found a significant difference in H3K4me2 levels between groups with low (%HDS ≤ 15) and those with high (%HDS > 15) DNA staining. This finding on flow cytometry was supported by Western blot analysis of pooled samples (n=10) **(Fig. 2a, b)**. In addition, we identified a positive correlation between H3K4me2 and %HDS (r = 0.47; $p < 0.001$) and, thus, proved the relationship between sperm immaturity and the epigenetic marker H3K4me2.

H3K4me2 is an indispensable element in the formation of the paternal chromatin, and it has been detected in the promoters of transcriptionally active housekeeping genes and the genes necessary for spermatogenesis[15]. Moreover, H3K4me2 is probably involved in chromatin opening during histone-to-protamine exchange[106]. Although a small amount of H3 remains in mature sperm, most of it, along with its modification, is removed during sperm elongation.

Therefore, samples with a high incidence of H3K4me2 probably contain a large number of spermatozoa with incomplete chromatin silencing and/or protamination, which ultimately reduces semen fertility. This information regarding H3K4me2 content could improve the diagnosis of male infertility and its treatment.

Further analysis showed that the H3K4me2 level was correlated with sperm concentration ($r = -0.570$; $p < 0.001$) and weakly with motility ($r = -0.25$; $p = 0.015$). H3K4me2 was significantly higher in A and OA than in N **(Fig. 2c)**. Interestingly, %HDS did not differ across samples of varying quality (N, A, OA), making H3K4me2 a better marker of chromatin condensation than %HDS **(Fig. 2d)**.

Incompletely packaged chromatin is more vulnerable to oxidative damage and DNA breaks than properly condensed chromatin. Many studies have declared a relationship between DNA fragmentation incidence and insufficiently condensed chromatin[107–110]. We found that %DFI increases with deteriorating sperm quality (N, A, and OA), but we did not find any relationship between %DFI and H3K4me2 incidence. We found a significant correlation between %DFI and %HDS, but the correlation was weak ($r = 0.2$; $p = 0.047$).

Altogether, studies regarding chromatin compactness have shown that incomplete protamination, the protamine ratio, and PTMs of residual histones are reliable prognostic markers for sperm quality assessment and ART success[111–113]. In this study, we introduced the first H3K4me2 as a useful marker of sperm quality, which seems to be a more accurate indicator of chromatin condensation than %HDS. Our findings are in accordance with previous observations of histone code-based sperm quality markers, indicating the future of assisted reproduction diagnostics[114–116]. The index of sperm histone PTMs, called the histone code, reflects the quality of the spermatozoa and the viability of future embryos[79,84,117]. The histone code is largely inherited *via* fertilization and is thereafter involved in early embryonic development[6,9,118–120]. Taken together, existing knowledge supplemented by our own observations emphasizes the significance of H3K4me2 as a marker of sperm quality.

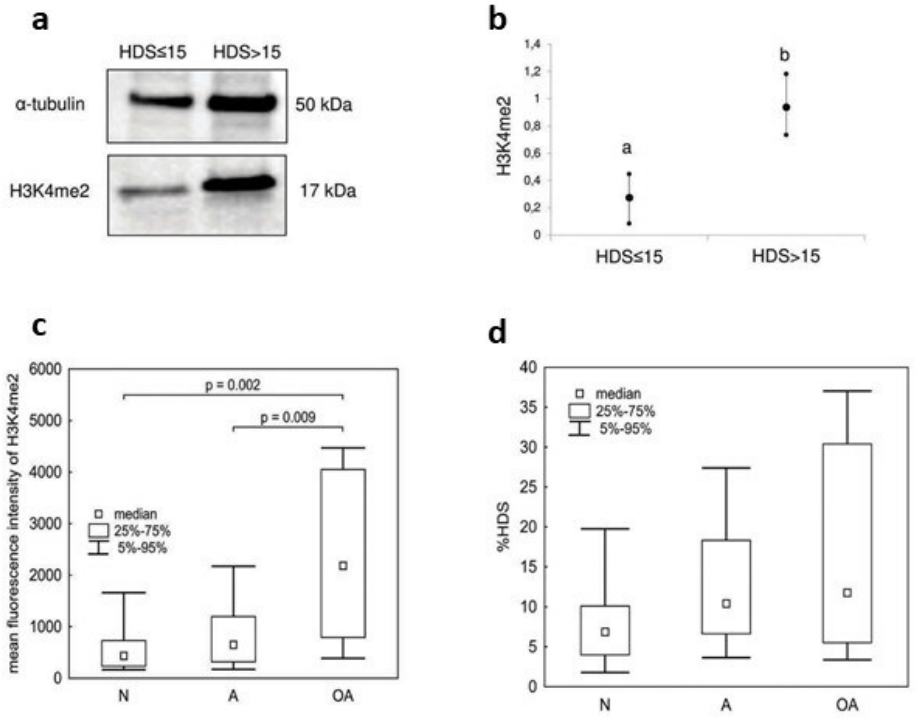


Figure 2. H3K4me2 occurrence in human spermatozoa of different qualities. (**a)** Western blot of H3K4me2 occurrence in high and low HDS samples (HDS≤15 and HDS≥15, respectively). (**b)** Western blot densitometry of H3K4me2 differences between high and low HDS samples. (**c)** Comparison of H3K4me2 fluorescence intensity measured by flow cytometry between normozoospermic (N), asthenozoospermic (A), and oligoasthenozoospermic (OA) samples. (**d)** Comparison of %HDS measured by flow cytometry between N, A, and OA samples. The data are expressed as medians and appropriate quartiles, and different superscripts indicate statistical significance (p<0.05). HDS, high DNA stainability index.

Conclusion

H3K4me2 demonstrates good ability to reflect chromatin immaturity, which makes H3K4me2 a very helpful marker for the diagnosis of male sub/infertility and possible failure of ART. However, tightness of the sperm nucleus, including nuclear protein markers, makes the reading of epigenetic information difficult. Therefore, further studies (*e.*g., correlation analyses with well-accessible sperm markers) and/or the development of diagnostic techniques are needed for further usage of these markers in ART.

These results have been published in a scientific 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. 2020. H3K4me2 accompanies chromatin immaturity in human spermatozoa: an epigenetic marker for sperm quality assessment. Syst Biol Reprod Med. 66(1):3-11. doi: 10.1080/19396368.2019.1666435. (**IF_{2020} = 3.061**)

4.2. Environmental pollutants: possible causes of male idiopathic infertility?

Due to the lack of gene expression, spermatozoa are extremely sensitive to environmental pollutants during their maturation in the epididymis. Some environmental pollutants, including the infamous endocrine disruptors, disrupt sex hormone activity and alter male and female reproduction. We hypothesize that in addition to altering sex hormone activity, endocrine disruptors could affect the sperm-wide protein acetylome and phosphorylome at very low (*i.e.,* nontoxic) doses and, thus, contribute to idiopathic male infertility. Moreover, we suggest that sperm PTMs may serve as a good marker for male sub/infertility diagnosis and can offer the possibility for treatment. In our experiment, we exposed adult male mice to the ubiquitous endocrine disruptor bisphenol S (BPS). While a high dosage of 100 μg/kg body weight (bw)/day caused histopathological changes in seminiferous tubules, a very low dosage of 0.001 μg/kg bw/day (dosage normally found in the environment) caused changes in the sperm-wide acetylome and phosphorylome. We used Western blotting for the quantification of differences between groups and mass spectrometry to identify possible targets of BPS-causing PTM changes.

Methods

Design of the experiment

All animal procedures were performed in accordance with the Protection of Animals against Cruelty (Act No. 246/1992), and the experiment was approved by the Welfare Advisory Committee at the Ministry of Education, Youth, and Sports of the Czech Republic.

Eight-week-old ICR male mice were randomized into the experimental groups and vehicle control group (0.1% ethanol; VC). The experimental mice were exposed to BPS to simulate real human BPS exposure. Therefore, very low concentrations of 0.001 (BPS1), low concentrations of 1.0 (BPS2), and high concentrations of 100 μg/kg bw/day (BPS3) BPS were administered through drinking water for 8 weeks (8–16 weeks of age). After the experiment, the mice were weighed and sacrificed, and the appropriate tissues were used for the analysis of the effects of BPS.

Sperm isolation and assessment

Spermatozoa were isolated from the cauda epididymis. The cauda was cut several times and placed into 0.5 mL of Whitten's medium, where spermatozoa were allowed to swim out for 30 min. Thereafter, sperm concentration and motility were evaluated using a Makler chamber and light microscope (Olympus CKX 41; Germany) equipped with a 10× objective (CAchN NA 0.25). The evaluation was performed by one person, and samples were blinded to avoid bias.

Western blot

Sperm samples of three individuals belonging to the same experimental group were pooled to detect the sperm acetylome and phosphorylome. Samples underwent protein lysis, Western blotting, and immunodetection of acetylated and phosphorylated proteins as described in the previous chapter. The resulting membranes were incubated with the following primary antibodies: anti-acetyl lysine antibodies (1:1,000; Abcam, UK), anti-phospho-tyrosine antibodies (1:1,000; Abcam, UK), and anti-acetylated α-tubulin antibodies (1:1,000; Sigma, USA). Anti-α-tubulin and anti-histone H3 antibodies (1:1,000; Cell Signaling Technology, USA) were used as the loading controls. The incubation with secondary antibodies was performed as described above.

nano-LC–MS

Nano-liquid chromatography coupled to tandem mass spectrometry (nano-LC–MS) was used to identify and quantify acetylated and phosphorylated proteins in mouse spermatozoa. Sperm protein lysates were prepared the same as for Western blotting (WB). The protein concentration was then estimated by the Bradford method[121] using Protein Assay Dye Reagent (Bio–Rad, France). The prepared samples were processed according to a filter-aided sample preparation (FASP) protocol described by Wiśniewski[122]. Briefly, approximately 200 µg of protein from each pooled sample was placed into an ultrafiltration device (Merck Millipore, USA), used for detergent removal, followed by trypsin digestion (37 °C, overnight). The resulting proteolytic peptide solutions were dried using a SpeedVac. Then, peptides were resolved in 100 µL of 5% ACN/0.1% HCOOH and filtered through a 0.22-µm PVDF syringe filter. The concentration of peptides was estimated by the Pierce™ Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific, USA), followed by dilution of peptides to 1 µg/µL and usage of 1 µg of peptide mixtures for nano-LC mass

spectrometry. LC/MS was performed using a NanoLC 425 UPLC system (Eksigent, CA, USA) coupled with a Triple TOF 5600 mass spectrometer (Sciex, USA). Protein identification was performed using the information-dependent analysis (IDA) method, and protein quantification was performed by the sequential window acquisition of all theoretical fragment ion spectra (SWATH) method. All acquired data were analyzed using Analyst TF 1.7 software (Sciex, USA) in the IDA and SWATH modes. For more details, see the supplementary material[123].

Statistical analysis

Data were analyzed as was described in the previous chapter.

Results and discussion

Bisphenols are infamous endocrine disruptors that disrupt hormonal homeostasis and, thus, indirectly cause, *e.g.,* obesity[124,125] and testis atrophy[126]. However, our experiment did not reveal an effect on hormone levels, body weight or relative testis weight. Therefore, endocrine disruptors, including bisphenols, can affect reproductive health through more subtle mechanisms related to cellular physiology, such as oxidative stress[127], disruption of certain enzyme activity[128], or alteration of DNA methylation[129]. Based on our results, we suggest another mechanism of bisphenol's action *via* alteration of protein acetylation and phosphorylation.

In our study, we followed the effects of three different BPS doses (0.001, 1.0 and 100 μg/kg bw/day) on male reproduction. The highest concentration of 100 μg/kg bw/day caused histopathological changes in seminiferous tubules, which was also confirmed in other studies[127,130,131]. The low concentration of 1.0 μg/kg bw/day seemed to have no harmful effect. However, the very low concentration of 0.001 μg/kg bw/day altered the sperm-wide acetylome and phosphorylome, presumably associated with decreased sperm motility **(Fig. 3).** The case of a lower concentration of a compound being more harmful than a higher concentration is illustrative of the nonlinear toxic effect[132]. This effect is well described for some endocrine disruptors, and we suggest that it is also the case for BPS[133].

We assume that changes in phosphorylation and acetylation may be responsible for motility decline in the experimental group administered 0.001 μg/kg bw/day BPS. Therefore, we identified protein candidates probably accountable for the decrease in phosphorylation signal

intensity in the 100-kDa band. One of the candidates is hexokinase, which is associated with male sterility in the absence of phosphorylation[134]. Another candidate is outer dense fiber protein 2, for which insufficient phosphorylation leads to motility failure[135]. Whereas loss of phosphorylation in specific proteins leads to severe problems, no data exist regarding the harmfulness of protein overphosphorylation, as is the case for the 85-kDa band.

Acetylation signal intensity loss was observed in the 50-kDa band, where α-tubulin, β-tubulin, and ATP synthase subunits occur. With specific antibodies against acetylated α-tubulin, we concluded that α-tubulin did not differ between groups **(Fig. 3d)**. However, acetylation also plays an important role in β-tubulin polymerization[136] and activation of ATP synthases[137], suggesting their possible participation in BPS-caused motility decrease.

To better understand when acetylation and phosphorylation of sperm proteins are disrupted, we analyzed the acetylome and phosphorylome of testicular tissue. We did not identify any differences between groups, and this finding supports our assumption that changes in sperm PTMs occur during sperm maturation in the epididymis.

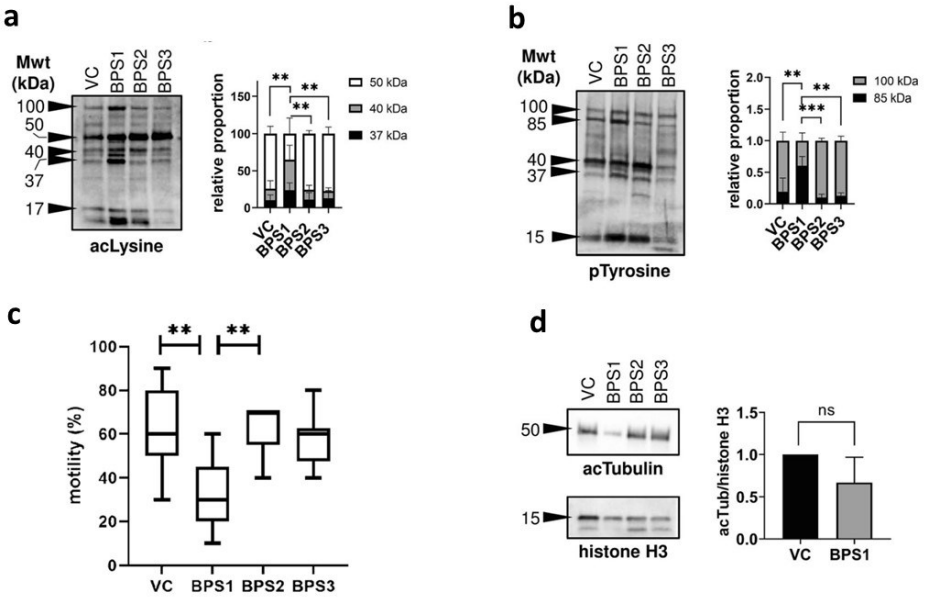


Figure 3. Effect of bisphenol S (BPS) on the sperm proteome and motility. (**a)** Representative Western blot of acetyl-lysine and densitometry analysis of major acetylated bands. Differences between groups treated with different BPS doses: BPS1 (0.001 μg/kg bw/day), BPS2 (1.0 μg/kg bw/day), BPS3 (100 μg/kg bw/day) and VC (vehicle control). (**b)** Representative Western blot of tyrosine phosphorylation and densitometry analysis of major phosphorylated bands. Differences

between groups BPS1, BPS2, BPS3, and VC. (**c)** Different percentages of motile spermatozoa within the BPS1, BPS2, BPS3, and VC groups. (**d)** Densitometric analysis of acetylated alfa-tubulin from BPS1 and VC. Differences were assessed by two-way ANOVA, followed by Tukey's multiple comparison test, and asterisks indicate statistical significance *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

Conclusion

This study revealed a novel mechanism through which environmental pollutants disrupt cell physiology. This mechanism is represented by PTM of proteins, and in this study, we indicate that environmental pollutants represented by BPS can alter the sperm acetylome and phosphorylome at very low (nontoxic) concentrations. PTM alterations lead to serious reproductive problems, such as decreased sperm motility, suggesting that environmental pollutants are strong candidates for causing idiopathic infertility. The acetylation and/or phosphorylation of certain proteins could serve as a marker of sperm sub/infertility and help facilitate the development of effective treatments.

These results have been published in a scientific journal with an impact factor and are attached as the appendix (A2).

Řimnáčová H., Štiavnická M., Moravec J., Chemek M., Kolinko Y., García-Álvarez O., Mouton P.R., Trejo A.M.C., Fenclová T., Eretová N., Hošek P., Klein P., Králíčková M., Petr J., Nevoral J. 2020. Low doses of Bisphenol S affect post-translational modifications of sperm proteins in male mice. Reprod Biol Endocrinol. 18(1):56. doi: 10.1186/s12958-020-00596-x. (**IF_{2020} = 5.211**)

4.3. Role of persulfidation, a redox PTM driven by H_2S, in male reproduction

Reactive oxygen, nitrogen and sulfur species (RONSS) control protein activity *via* PTMs of target proteins. RONSS-derived PTMs are called redox PTMs, and they arise on cysteine's sulfhydryl group (-SH). Cysteine modifications participate in redox balance maintenance, which is extremely important and likewise challenging for spermatozoon for the following reasons: i) it lacks antioxidants, which are naturally removed from spermatozoon with cytoplasm during sperm development, ii) there is a significant production of free radicals due to the high metabolic rate, iii) sperm membrane lipids are extremely vulnerable to peroxidation *via* ROS, and iv) the spermatozoon possesses a limited capacity for oxidative damage repair since it is transcriptionally and translationally silent. Accordingly, redox PTMs play an irreplaceable role in redox balance maintenance, controlling protein activity and capacitation.

In this study, we focused on persulfidation ($-(S)_nH$), one of the redox PTMs. While most of the redox PTMs originate from the oxidation of -SH (*e.g.,* nitrosylation, sulfenylation, disulfide bonds), $-(S)_nH$ is formed by reduction and prevents cysteine hyperoxidation and protein damage. Most $-(S)_nH$ is caused by hydrogen sulfide (H_2S), an endogenously produced gasotransmitter released enzymatically in cells. Our research group described the presence of cystathionine β-synthase, cystathionine γ-lyase, and mercaptosulfuryl transferase, all of the currently known H_2S-releasing enzymes, in mammalian spermatozoa. In accordance with enzyme detection, the endogenous production of H_2S *via* these enzymes was described. Finally, proteins undergoing $-(S)_nH$ were detected in spermatozoa. The purpose of $-(S)_nH$ in sperm physiology and its potential role in male reproduction are discussed, as well as the possibility of its use in ART.

Methods

Sperm samples

Two model organisms were used for the study, namely, humans and mice (strain C57Bl/6). Mouse spermatozoa isolated from the cauda epididymis were allowed to swim out to human tubal fluid medium supplemented with HEPES (HTF-HEPES). The medium was enriched with 0.4% bovine serum albumin (BSA) in the case of capacitating medium. Human spermatozoa were allowed to swim up from ejaculates into HTF-HEPES medium, which

was placed over the ejaculate, for 2.5 h in a 37 °C water bath. In the case of the capacitated group, HTF-HEPES medium was enriched with 0.3% BSA. All handling of human samples followed the World Health Organization (WHO) manual (2010)[138]. Thereafter, all samples were processed in the assays described below.

In situ detection of persulfidation in spermatozoa

Persulfidated (-$(S)_nH$) proteins were visualized in spermatozoa using a modified switch assay. First, spermatozoa were subjected to a LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen Life Technologies, USA) as previously described[139]. Persulfidation (-$(S)_nH$) and free thiols (-SH) were distinguished in accordance with a previous study[140], with several modifications. Briefly, free thiols of spermatozoa were blocked by 20 mM methyl methanethiosulfonate (MMTS, Sigma–Aldrich, USA) dissolved in HEN buffer (250 mM HEPES-NaOH (pH 8), 1 mM EDTA, and 0.1 mM neocuproine) for 60 min at 38 °C on a shaker. After blocking, the sperm suspension was washed three times with PBS for 10 min on a shaker and centrifuged (300 g). Presumed -$(S)_nH$ in spermatozoa was stained with 0.04 µM 6-iodoacetamidofluorescein (6-IAF, Thermo Fisher, USA) for 15 min at room temperature and fixed in 3.2% paraformaldehyde for 10 min. The prepared samples were analyzed using a BD FACS Aria Fusion Cell Analyzer (Becton Dickinson, Prague, Czech Republic) for flow cytometry. Data were collected from 5,000 events. LIVE/DEAD Fixable Dead Cell Stain and 6-IAF were excited by 405- and 488-nm lasers and detected with 450/50 and 530/30 bandpass filters. Acquired data were analyzed using FlowJo software (Becton Dickinson, Prague, Czech Republic). Alternatively, spermatozoa were added to coverslips, and -$(S)_nH$ was visualized *in situ* via an Olympus IX83 fluorescence microscope (Olympus, Germany).

Biotin switch method and pull-down assay

Detection of persulfidated (-$(S)_nH$) proteins in lysate was performed as previously described[140] with modifications. Approximately 20 million spermatozoa or 100 mg of tissue (brain, liver and testis) was lysed in 100 µL of HENS buffer (250 mM HEPES-NaOH (pH 8), 1 mM EDTA, 0.1 mM neocuproine, 1% SDS) and incubated on a shaker for 30 min. Then, the lysate was centrifuged (10,000 g), 50 µL of supernatant was mixed with 150 µL of HEN buffer, and 0.38 µL of MMTS was added (reaching a final concentration of 20 mM). Free -SH in protein lysate was blocked for 20 min at 50 °C on a shaker. The residue of

MMTS was then removed by ethyl acetate extraction, followed by vortexing three times, brief centrifugation, ethyl acetate removal by a pipette, and vacuum evaporation of the rest of the solution. The samples were labeled with 3.3 mM EZ-link iodoacetyl-PEG_2-biotin (ThermoFisher, USA) overnight at 4 °C on a shaker, and -$(S)_nH$ was selectively switched *via* this modified biotin. To validate the specificity of the method used, we prepared three specifically treated groups to detect -$(S)_nH$ + -SH (no MMTS treatment), -$(S)_nH$ only (MMTS treatment), and naturally biotinylated proteins (nonalkylated control) **(Fig. 4a, b)**. Iodoacetamide (Sigma–Aldrich, USA) was used as a negative control omitting biotin. The aliquot of treated proteins was diluted in Laemmli loading buffer under reducing agent-free conditions. Samples and controls were separated by SDS-ELFO and visualized by Western blot analysis using HRP-conjugated streptavidin (Sigma–Aldrich, USA; 1:11,000) and chemiluminescence detection as mentioned above. As an alternative to Western blotting, other aliquots were loaded onto streptavidin-coated agarose beads (Millipore, USA) and incubated overnight at 4 °C on a shaker. Beads were treated with 100 mM β-mercaptoethanol in 4% SDS, and primary persulfidated proteins were eluted **(Fig. 4a)**. The purified samples were processed for nano-LC–MS as described below.

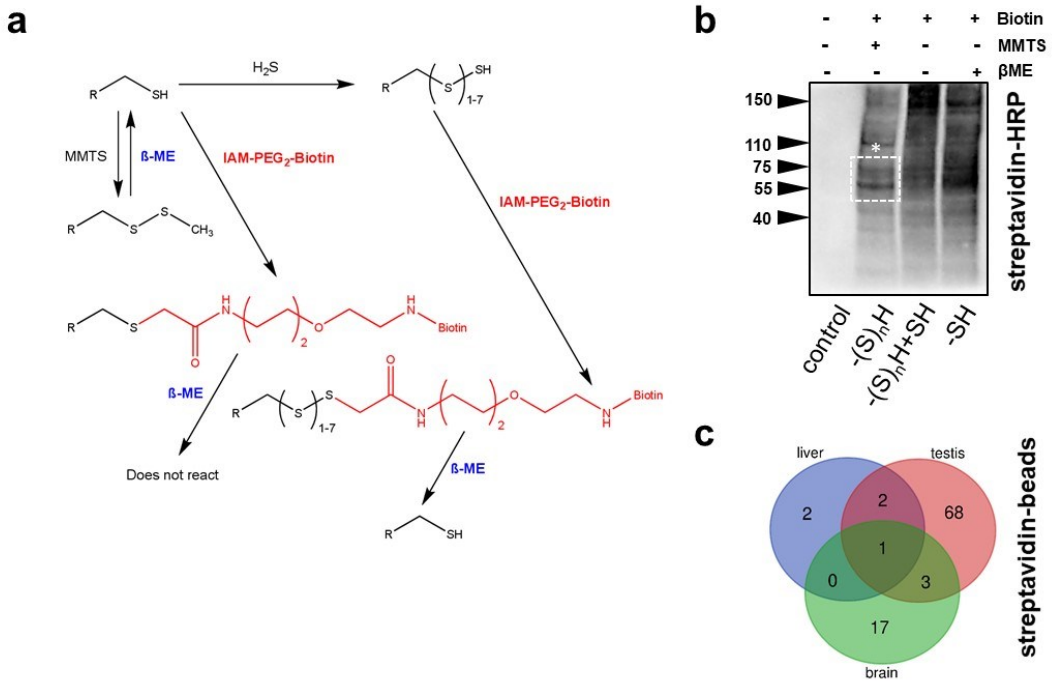


Figure 4. Persulfidation in mouse tissues with emphasis on the testis. **(a)** Principle of selective detection of persulfidation (-$(S)_nH$) using the thiol-selective binding ability of S-methyl methanethiosulfonate (MMTS) and the binding affinity of IAM-PEG-biotin to thiols (-SH). **(b)** β-Mercaptoethanol (β-ME), a reducing agent, was used to eliminate persulfide-biotin bonds and selectively detect free -SH. Bands in the size range 55–75 kDa (*) belonging to abundant proteins modified by -$(S)_nH$. **(c)** Alternatively, selectively labeled -$(S)_nH$ with IAM-PEG-biotin was loaded on streptavidin-coated agarose beads. The eluted native proteins were digested and detected by nano-LC–MS. Liver, brain, and testicular tissues were processed *via* pulldown assays and nano-LC–MS detection. Persulfidated protein content was compared, and the results are expressed in a Venn diagram.

nano-LC–MS

Nano liquid chromatography coupled to tandem mass spectrometry (nano-LC–MS) was used to identify and quantify protein persulfidation (-$(S)_nH$) from mouse testis and spermatozoa. For this purpose, samples from the biotin-streptavidin pull-down assay were used. The following procedure was performed as described in the previous chapter.

Na_2S treatment of spermatozoa

Isolated spermatozoa were separated into groups and diluted 1:1 with HTF-HEPES supplemented with 0.4% BSA and the appropriate concentrations of $Na_2S.9H_2O$ (Sigma–Aldrich, USA). Importantly, $Na_2S.9H_2O$ solution was prepared shortly before its use. Sperm treatment was performed 30 min after sperm isolation. In the case of the experiment, which tested a concentration series, the final $Na_2S.9H_2O$ concentrations were as follows: 0, 5, 15, 50, and 150 µM. Then, spermatozoa were incubated in a thermoblock at 37 °C and checked three times: T0 – immediately after isolation, T2 – two hours after isolation, and T5 – five hours after isolation. In the case of the experiment, with the selected concentration of 5 µM used for IVF, HTF without HEPES was used, and spermatozoa were incubated under mineral oil (Sigma–Aldrich, USA) in an incubator (5% CO_2, 37 °C).

Mitochondrial superoxide determination by flow cytometry

The detection of mitochondrial superoxide was performed using MitoSOX (Thermo Fisher, USA) in combination with SYTOX Green vitality staining (Thermo Fisher, USA). After $Na_2S.9H_2O$ treatment, spermatozoa were washed, diluted in HTF-HEPES and incubated with MitoSOX (2 µM) and SYTOX Green (0.01 µM) for 10 min. Thereafter, spermatozoa were washed and analyzed by BD-FACS-Aria flow cytometry. Excitation of both stains was performed with a blue laser (488 nm), and MitoSOX and SYTOX fluorescence were detected with 700/54 and 537/32 BP filters, respectively.

***In vitro* fertilization**

After sperm isolation, spermatozoa were allowed to capacitate for 1 h in an incubator (5% CO2, 37 °C), whereas $Na_2S.9H_2O$ treatment (5 µM) was carried out within the last 30 min. Meanwhile, hormonally stimulated 8- to 12-week-old females (by equine and human chorionic gonadotropin administration (10 I.U., respectively) 48 and 72 h apart) were euthanized by cervical dislocation, and cumulus-oocyte complexes (COCs) were isolated from the ampulla. COCs were then coincubated with the capacitated sperm at a concentration

of $10x10^6$ sperm mL^{-1} for 5.5 h in the same medium and under the same conditions applied for capacitation. Thereafter, presumed zygotes were cultured in EmbryoMax KSOM Mouse Embryo medium (Millipore, USA) supplemented with 0.1% BSA until two-cell stage embryos were obtained. Alternatively, the resulting zygotes were fixed in 4% paraformaldehyde supplemented with 0.1% polyvinyl alcohol after 5.5 h of incubation, and zygotes were used for differential staining of paternal pronuclei.

Immunocytochemistry of zygotes

Zygotes resulting from IVF with $Na_2S.9H_2O$-treated and untreated (control) spermatozoa were used for Lamin B1 staining for evaluation of pronuclei diametral. Fixed zygotes were permeabilized in PBS solution containing 0.04% Triton X-100 and 0.3% Tween 20 for 10 min and blocked in blocking solution (PBS, 5% BSA, 0.1% Tween 20) for an hour. Then, zygotes were incubated with anti-Lamin B1 antibodies (1:200; Abcam, UK) diluted in washing solution (1% BSA, 0.1% Tween 20) for an hour, followed by three washes and incubation with secondary antibodies (1:200; Abcam, UK) for an hour. Stained zygotes were mounted on the slides by Vectashield containing DAPI (Vector Laboratories, USA) and visualized via an Olympus IX83 fluorescence microscope (Olympus, Germany).

Statistical analysis

Data were analyzed as aforementioned.

Results and Discussion

The persulfidation ($-(S)_nH$) arises from the reducing reaction, and, therefore, it could be crucial for maintaining redox balance in poorly equipped spermatozoa for oxidative stress defense. Many proteins are regulated by oxidative PTMs in male reproduction[57,141], such as S-nitrosylation and S-sulfenylation. This fact makes $-(S)_nH$ even more important because it prevents cysteine from undergoing hyperoxidation and, thus, prevents redox signaling pathway disruption and protein deactivation. Surprisingly, $-(S)_nH$ has not been investigated to date. This study identifies the protein $-(S)_nH$ in mouse testes and human spermatozoa and suggests a possible use of H_2S donation in ART.

First, we performed quantitative and qualitative analyses of $-(S)_nH$ in the mouse testis within other redox PTMs, namely, S-nitrosylation and S-sulfenylation (data are presented in the paper). We observed that proteins subjected to $-(S)_nH$ ranged from 40 to 150 kDa **(Fig. 4b)**.

Persulfidated proteins were then identified using the pull-down assay followed by nano-LC–MS **(Fig. 4c)**. We compared -$(S)_nH$ from the testis with that from the brain and liver, tissues where -$(S)_nH$ was previously described. We found proteins that underwent -$(S)_nH$ across the tissues, but we found 68 proteins that were modified only in the testis. These findings suggest that -$(S)_nH$ targets proteins specifically in the testis, even if these are widely expressed proteins.

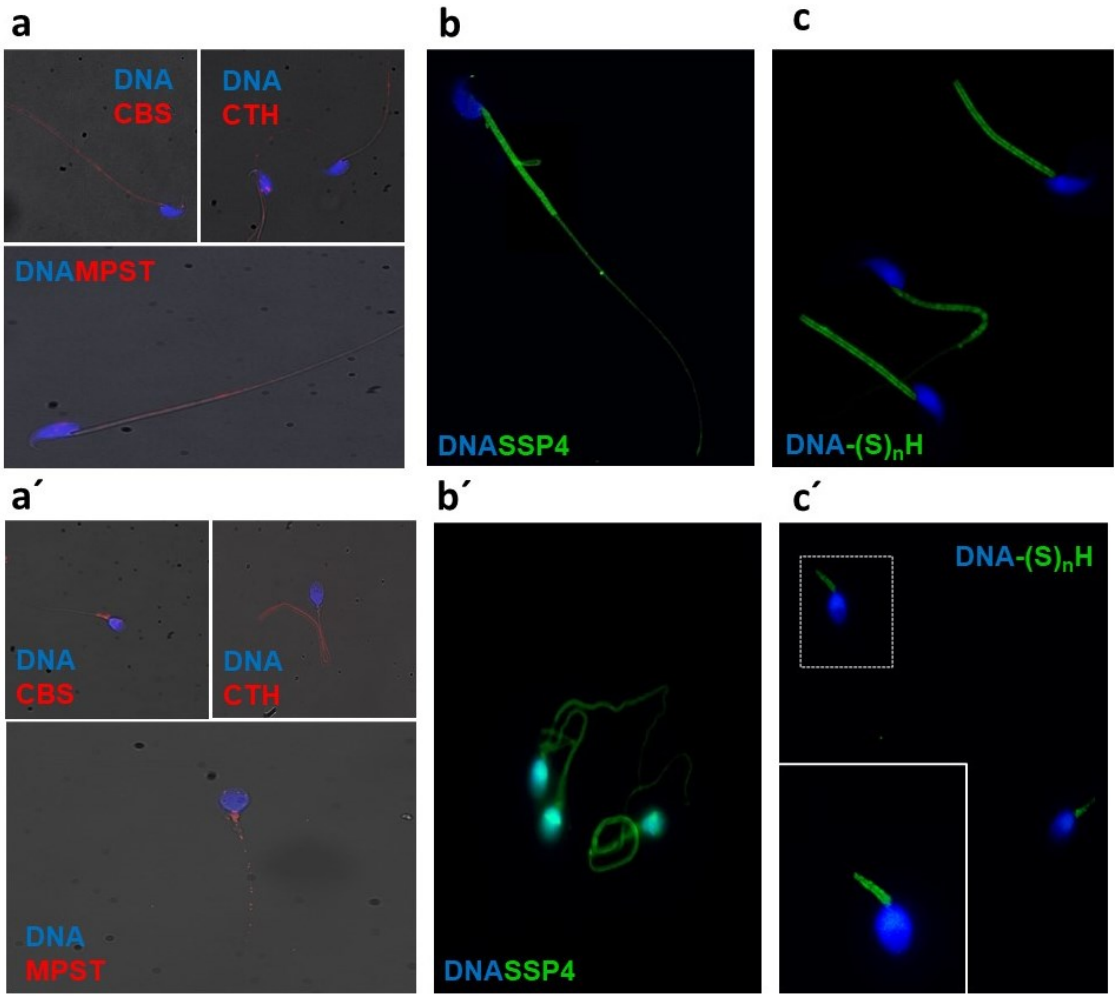
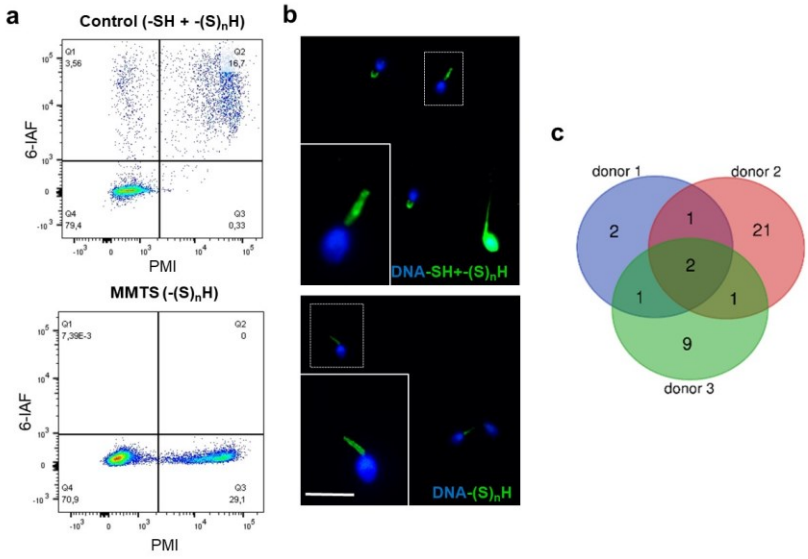


Figure 5. H_2S production and persulfidation (-$(S)_nH$) in **(a – c)** mouse and **(a´ – c´)** human spermatozoa. **(a, a´)** immunocytochemistry of cystathionine β-synthase (CBS), cystathionine γ-lyase (CTH) and 3-mercaptopyruvate sulfurtransferase (MPST). **(b, b´)** Localization of H_2S production by Sulfane Sulfur Probe 4 (SSP4) and -$(S)_nH$ **(c, c´)**. Magnitude of human spermatozoa with persulfidation in the midpiece in the white rectangle.

We postulate a high content of -$(S)_nH$ in spermatozoa based on our finding of high -$(S)_nH$ abundance in mouse testis. Indeed, the presence of -$(S)_nH$ could clarify how spermatozoa control protein activity and resist oxidative stress despite their limitations in protein production. Therefore, we focused on spermatozoa in the following experiments, where we detected -$(S)_nH$ *in situ* in mouse and human spermatozoa. We observed that -$(S)_nH$ was strictly located in the sperm midpiece **(Fig. 5)**, which highly corresponds to H_2S occurrence and the location of its enzymes[61]. Our findings support those of previous studies, which consider -$(S)_nH$ in tissue to be the result of H_2S action[142]. To learn more about the role of -$(S)_nH$ in sperm physiology, we costained -$(S)_nH$ with a live/dead staining kit in human spermatozoa **(Fig. 6)**. We analyzed spermatozoa from normozoospermic donors by flow cytometry. We divided each sample into the positive control group, where we detected -SH + -$(S)_nH$, and the MMTS group, where we detected -(S)nH– only. Surprisingly, we found that spermatozoa are -$(S)_nH$ independent of live/dead status (MMTS group), and –$(S)_nH$ is located exclusively in the sperm midpiece **(Fig. 6a, b)**.

Persulfidation located specifically in the mitochondrial sheath may play an important role in sperm metabolism and maintenance of redox balance. We supported this statement by identifying -$(S)_nH$ using mass spectrometry. In most cases, the identified proteins were associated with mitochondrial metabolism and flagellar movement **(Fig. 6d)**. Some of these proteins have been reported to undergo -$(S)_nH$, including glyceraldehyde-3-phosphate dehydrogenase, tubulin[140] and L-lactate dehydrogenase[57], but we were the first group to observe that these proteins were persulfidated in human spermatozoa. Some -$(S)_nH$ targets were previously discovered to be S-nitrosylated, including A-kinase anchor protein, heat shock protein and semenogelin[57], which supports the idea that S-nitrosylation serves as a -$(S)_nH$ precursor[50,60,143]. All these results demonstrate that spermatozoa contain many proteins containing reactive cysteine, while these proteins can be easily switched on and off by these redox PTMs.



d

Entry	Entry name	Proteins names	Length (AAA)	Mass (Da)
Q5JQC9	**AKAP4_HUMAN**	**A-kinase anchor protein**	**854**	**94,477**
Q14556	**G3PT_HUMAN**	**Glyceraldehyde-3-phosphate dehydrogenase testis-specific**	**408**	**44,501**
P07864	**LDHC_HUMAN**	**L-lactate dehydrogenase C chain**	**332**	**36,311**
Q02383	**SEMG2_HUMAN**	**Semenogelin-2**	**582**	**65,444**
Q6PEY2	**TBA3E_HUMAN**	**Tubulin alpha-3E chain**	**450**	**49,859**
P07288	KLK3_HUMAN	Prostate-specific antigen	261	28,741
Q9BQE3	TBA1C_HUMAN	Tubulin alpha-1C chain	449	49,895
Q13275	SEM3F_HUMAN	Semaphorin-3F	785	88,381
Q9BUN1	MENT_HUMAN	Protein MENT	341	36,769
P21266	GSTM3_HUMAN	Glutathione S-transferase Mu 3	225	26,560
P41229	KDM5C_HUMAN	Lysine-specific demethylase 5C	1560	175,720
P14618	KPYM_HUMAN	Pyruvate kinase PKM	531	57,937
Q5JX71	F209A_HUMAN	Protein FAM209A	171	19,603
P10323	ACRO_HUMAN	Acrosin	421	45,847
P06733	ENOA_HUMAN	Alpha-enolase	434	47,169
P01266	THYG_HUMAN	Thyroglobulin	2768	304,790
P37802	TAGL2_HUMAN	Transgelin-2	199	22,391
P98164	LRP2_HUMAN	Low-density lipoprotein receptor-related protein 2	4655	521,958
P04075	ALDOA_HUMAN	Fructose-bisphosphate aldolase A	364	39,420
Q9NY87	SPNXC_HUMAN	Sperm protein associated with the nucleus on the X chromosome C	97	10,982
Q9HCE0	EPG5_HUMAN	Ectopic P granules protein 5 homolog	2579	292,481
Q9Y5B0	CTDP1_HUMAN	RNA polymerase II subunit A C-terminal domain phosphatase	961	104,399
P49916	DNLI3_HUMAN	DNA ligase 3 OS=Homo sapiens	1009	112,907
Q9BS86	ZPBP1_HUMAN	Zona pellucida-binding protein 1	351	40,142
Q8IXK0	PHC2_HUMAN	Polyhomeotic-like protein 2	858	90,713
Q67FW5	B3GNL_HUMAN	UDP-GlcNAc:betaGal beta-1 3-N-acetylglucosaminyltransferase-like protein 1	361	40,713
Q9UPI3	FLVC2_HUMAN	Feline leukemia virus subgroup C receptor-related protein 2	526	57,241
Q9H0C2	ADT4_HUMAN	ADP/ATP translocase 4	315	35,022
P10909	CLUS_HUMAN	Clusterin	449	52,495
P46439	GSTM5_HUMAN	Glutathione S-transferase Mu 5	218	25,675
Q9BXN6	SPNXD_HUMAN	Sperm protein associated with the nucleus on the X chromosome D	97	11,029
P02768	ALBU_HUMAN	Albumin OS=Homo sapiens	609	69,367
Q5VTE0	EF1A3_HUMAN	Putative elongation factor 1-alpha-like 3	462	50,185
Q6UW49	SPESP_HUMAN	Sperm equatorial segment protein 1	350	38,931
Q9Y696	CLIC4_HUMAN	Chloride intracellular channel protein 4	253	28,772
P04279	SEMG1_HUMAN	Semenogelin-1	462	52,131
P54652	HSP72_HUMAN	Heat shock-related 70 kDa protein 2	639	70,021

Figure 6. Free thiols and persulfidation analysis of proteins in human spermatozoa. **(a)** Flow cytometry of thiols (-SH) and persulfidation (-(S)$_n$H) in spermatozoa due to 6-iodoacetamidofluorescein (6-IAF) staining without (control) and with (MMTS) blocking of free thiols. The dot plot shows the separation of sperm subpopulations according to plasma membrane integrity (PMI) and 6-IAF signal intensity. (**b**) Representative images of -SH and -(S)$_n$H detection *in situ* showing 6-IAF staining patterns in the MMTS and control groups. The white rectangle indicates the -(S)$_n$H-assumed signal in the midpiece of emphasized spermatozoa (scale bar: 10 µm). (**c**) Venn diagram shows 37 persulfidated proteins identified in three independent donors by nano-LC–MS. (**d)** All detected persulfidated proteins are shown in the table. Persulfidated proteins that matched in at least two donors are shown in bold.

Based on our results, we consider a possible therapeutic effect of the H_2S donor on sperm during *in vitro* manipulation. We tested various concentrations of $Na_2S.9H_2O$ (Na_2S), a H_2S donor (0, 5, 15, 50, and 150 μM), on sperm motility, acrosome integrity, and the ability of disulfide bond reduction, along with capacitation **(Fig. 7a-c)**. We found that a concentration of 5 μM had no adverse effect on sperm motility and acrosome integrity during a five-hour *in vitro* incubation. Therefore, we consider this to be a physiological dose for use in further experiments **(Fig. 7a, b)**. We then found that Na_2S can reduce cysteine-driven PTMs at a concentration of 150 μM, and lower concentrations showed a reducing effect; however, the effect was not significant **(Fig. 7c)**. Our suggested H_2S dose is comparable to those previously used for spermatozoa treatment[102,103].

We pretreated sperm with capacitating medium supplemented with 5 μM Na_2S, and we then analyzed them *via* flow cytometry and Western blotting. Concurrently, we used spermatozoa for IVF and embryo production. Spermatozoa were subjected to superoxide staining produced by sperm mitochondria **(Fig. 7d)**. Pretreatment with the H_2S donor tended to decrease mitochondrial superoxide production; however, the difference was not statistically significant **(Fig. 7e)**.

We consider $-(S)_nH$ to be a possible H_2S mode of action; therefore, we performed selective labeling and detection by WB **(Fig. 7f)**. In lysates of sperm associated with different treatments and sampling times, we detected bands of persulfidated proteins from 150 to 16 kDa. Although we did not observe any pattern changes associated with treatment type and time, we suggest that individual band intensity changes were caused by the treatment; however, further analysis should be performed to confirm this. Even though we did not find any statistically substantiated evidence of an H_2S donor effect directly on spermatozoa, we surprisingly observed a significant effect of sperm pretreatment on the fertility rate **(Fig. 7g)**. Although we assumed that the reduction of protamine disulfide bonds is responsible for enhancing the fertility rate, we did not observe any difference in the dynamics of paternal pronuclear development, as its diameter was not changed **(Fig. 7h)**.

However, there are only a few studies indicating the therapeutic effect of H_2S supplementation on spermatozoa and testicular tissue[102,103,144], but the mechanism of H_2S action remains unknown. Understanding how H_2S affects spermatozoa and how H_2S-treated

spermatozoa are related to an improved fertility rate can have interesting implications for ART. Therefore, further study is needed to achieve a better understanding, knowledge transfer, modulation of sperm viability, and characterization of crucial proteins.

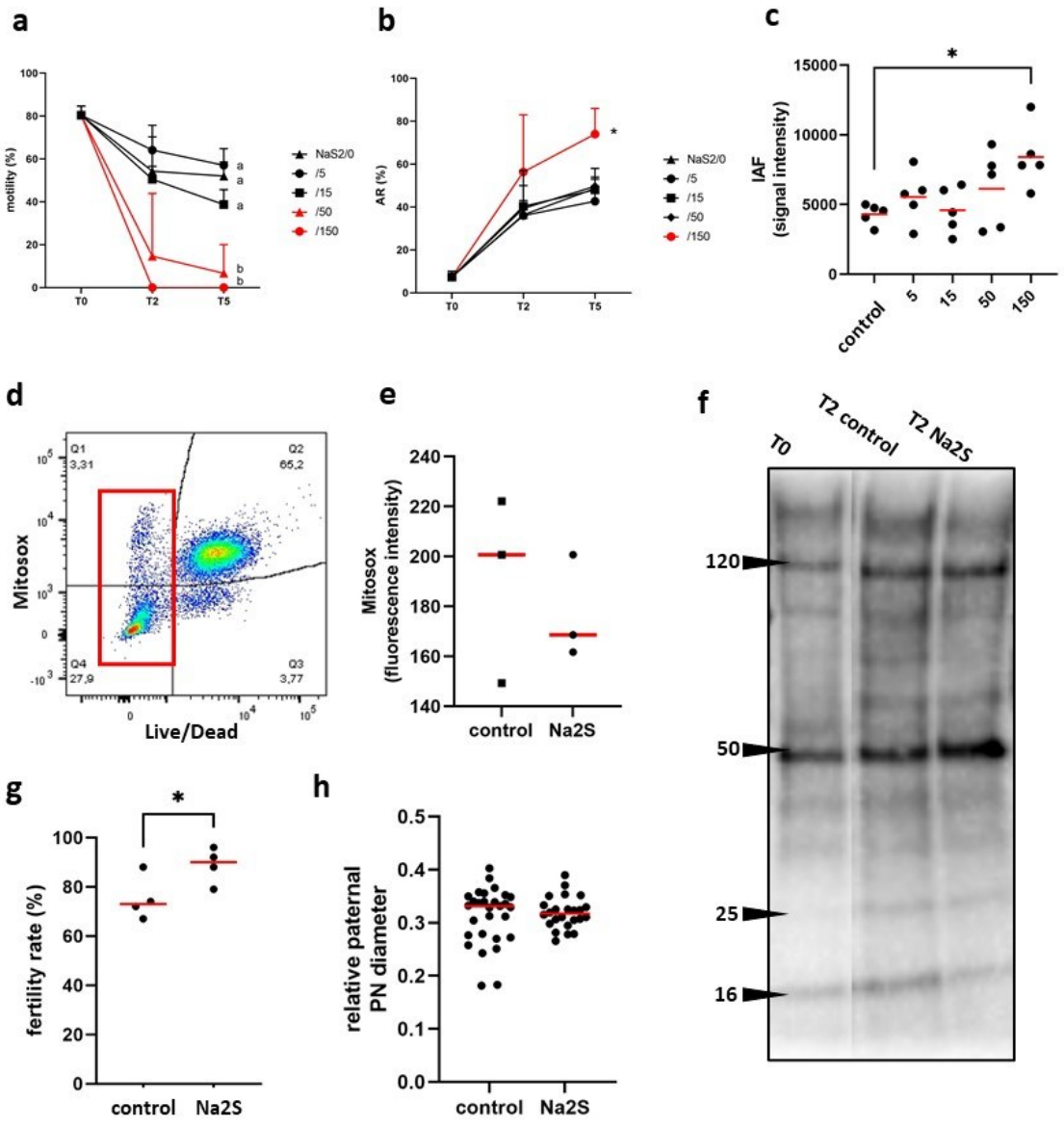


Figure 7. Spermatozoa treatment by different concentrations of H_2S donor (Na_2S). Test of the Na_2S concentration series, 0, 5, 15, 50, and 150 µM, on **(a)** sperm motility, **(b)** acrosome integrity, and **(c)** reduction ability over time. Concentrations with a harmful effect on spermatozoa are highlighted in red and considered to be toxic. **(d)** Sperm population separation is based on MitoSox/Sytox staining, where a population of viable spermatozoa is in the red rectangle. **(e)** MitoSox intensity differences between the groups. **(f)** Protein persulfidation detection by WB according to treatment time and

group. **(g)** Fertility rate differences between embryos raised from pretreated (Na_2S) and untreated (control) spermatozoa. **(h)** Relative paternal pronuclei (PN) diametral differences between the Na_2S and control groups. Different abbreviations or asterisks show statistically significant differences between groups ($p = 0.05$). In the figure, 0, 5, 15, 50 and 150 represent the Na_2S concentration series, and T0, T2 and T5 represent the times after sperm isolation, where T0, T2, and T5 represent 30 min, 2 hours, and 5 hours after sperm isolation, respectively.

Conclusions

Knowledge of the role of $-(S)_nH$ in mammalian spermatozoa is still preliminary; nevertheless, we can assume that $-(S)_nH$ acts as a protein modulator and, consequently, as a protecting agent. We consider the utility of our findings in clinical applications, *e.g.,* cryopreservation, *in vitro* fertilization, and preparation of insemination dosages. These approaches and their improvement increase the quality and survival of spermatozoa and, finally, ART outcomes. Although the positive effects of different H_2S donors have already been published, the mechanism of their action has remained unknown until now.

These results (except results from figure 7.) have been under reviewed in a scientific journal with an impact factor and are attached as the appendix (A3).

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5. Summary and perspectives

In vivo fertilization conditions create a sophisticated sperm selection mechanism that is difficult to simulate under *in vitro* conditions. However, research on sperm physiology can lead to improved *in vitro* conditions and the identification of new sperm quality markers, which can significantly improve ART. From this point of view, we performed three studies:

1. We studied histone H3 di-methylated on lysine 4 (H3K4me2) and nominated it as a suitable indicator of sperm chromatin maturity. The urgency to involve histone markers in sperm diagnosis has enhanced recent studies, which have shown that histone PTMs could be altered by environmental factors, such as diet, lifestyle, and environmental pollutants. These alterations of histone PTMs dysregulate embryonic gene expression and lead to developmental defects. Apparently, traditional sperm parameters (concentration, motility, and morphology) are insufficient for sperm diagnosis, and novel parameters, such as histone PTMs, are needed for ART improvement.
2. Following the significance of sperm PTMs, we outlined a novel method of assessing the harmfulness of environmental pollutants. We consider that bisphenol S, a ubiquitous environmental pollutant, can alter the PTMs of sperm nonnuclear proteins. Our study demonstrated changes in the acetylome and phosphorylome of sperm proteins, accompanied by motility decline. Based on our results, sperm PTMs represent advanced markers of the environmental impact on male fertility and serve as a helpful marker for sub/infertility diagnosis.
3. We described the presence of persulfidation (-$(S)_nH$) in spermatozoa and suggested its role in sperm physiology. This unique PTM, arising from the sulfhydryl group of cysteine, can protect proteins from hyperoxidation and damage. Moreover, H_2S and persulfidation contribute to the cell redox balance. Therefore, there is a suspicion that its supplementation *in vitro* can allow sperm to capacitate instead of being harmed by oxidative stress. Taken together, the results indicate that H_2S is a unique molecule with physiological action and is possibly supplemented under *in vitro* conditions. These features make H_2S an extremely interesting candidate for use in ART, which is still struggling, due to many limitations, with failures.

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7. APPENDIX

A1

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