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Ph.D. Thesis Abstract

Posttranslational modifications of nuclear and nonnuclear proteins in spermatozoa

Posttranslační modifikace jaderných a nejaderných proteinů ve spermiích

Modificaciones postraduccionales de proteínas nucleares y no nucleares en los espermatozoides

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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.

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.

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 acetilización 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.*

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1. Overview

There are an increasing number of couples in Western countries who cannot conceive naturally and need the help of reproductive clinics[1,2]. 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[3–6]. 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[6,7]. 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[8]. 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).

2. Methods

Semen samples

In H3K4me2 study, spermatozoa were washed from seminal plasma and kept in culture medium (Biggers-Whitten Whittingham medium) in an incubator (37 °C, 5% CO_2). In the following studies, the sperm of mice (strains C57Bl/6 or ICR) and humans were processed as follow. 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)[1].

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. SCSA was performed according to Evenson and Jost[2]. 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 for three minutes. 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[3]. 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

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 3% BSA in TBS with 0.05% Tween-20 (TBS-T) for 60 min at room temperature. Following antibodies were used in relevant experiment: rabbit polyclonal anti-H3K4me2 (1:1,000; Abcam, UK), anti-acetyl lysine antibodies (1:1,000; Abcam, UK), anti-phospho-tyrosine antibodies (1:1,000; Abcam, UK). Incubation with primary antibodies was overnight at 4 °C.. 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 $\leq$ 0.05, .01, .001, and .0001 were considered statistically significant and are indicated with asterisks (*), (**), (***), and (****), respectively.

Design of the Bisphenol S experiment

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.

nano-LC–MS

Depending on the experiment, appropriate tissue was collected for complete proteomic analysis. Nano-liquid chromatography-MS (nano-LC-MS) was used for protein identification and quantification, as described previously[4].

***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[5]. Persulfidation ($-(S)_nH$) and free thiols (-SH) were distinguished in accordance with a previous study[6], with several modifications.

Biotin switch method and pull-down assay

Detection of persulfidated ($-(S)_nH$) proteins in lysate was performed as previously described[6] with modifications. Approximately 20 million spermatozoa or 100 mg of tissue (brain, liver and testis) was used.

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 Na2S.9H2O 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% CO_2, 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).

3. 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.

4. Overview of our results

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

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) (n=22), asthenozoospermic (A) (n=63), and oligoasthenozoospermic (OA) (n=14). 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.

Results and Discussion

We proved the relationship between sperm immaturity and the epigenetic marker H3K4me2 ($r = 0.47$; $p < 0.001$) **(Fig. 2a, b)**. 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[30]. Moreover, H3K4me2 is probably involved in chromatin opening during histone-to-protamine exchange[31]. 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[32–35]. 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[36–38]. 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. 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. Our findings are in accordance with previous observations of histone code-based sperm quality markers, indicating the future of assisted reproduction diagnostics[39–41]. The index of sperm histone PTMs, called the histone code, reflects the quality of the spermatozoa and the viability of future embryos[4,7,42]. The histone code is largely inherited *via* fertilization and is thereafter involved in early embryonic development[43–47]. Taken together, existing knowledge supplemented by our own observations emphasizes the significance of H3K4me2 as a marker of sperm quality.

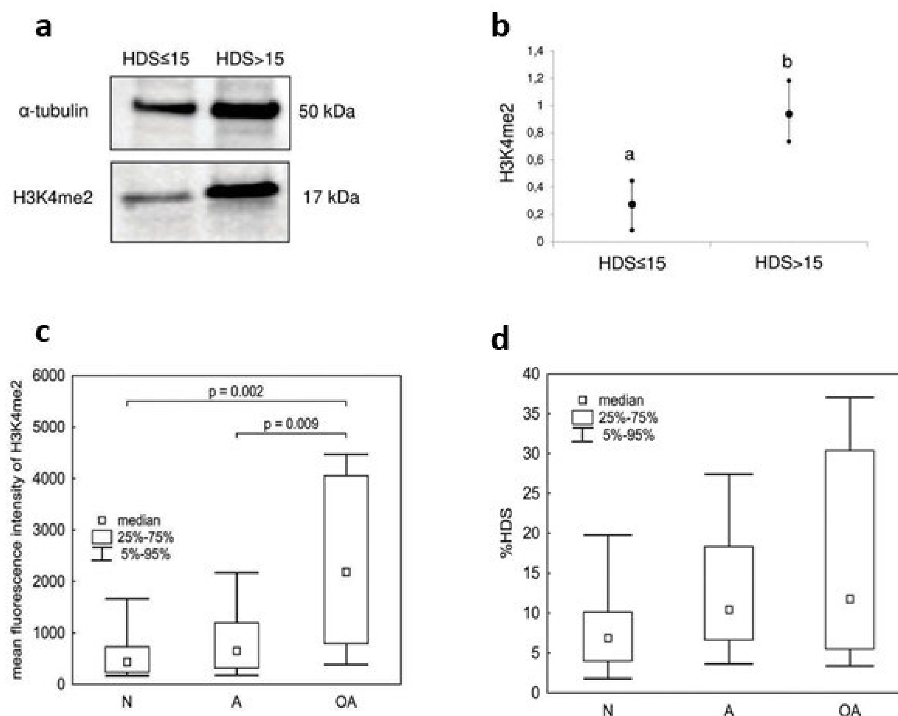

Figure 2. H3K4me2 occurrence in human spermatozoa of different qualities. (**a**) Western blot of H3K4me2 occurrence in high and low HDS samples (HDS≤15 and HDS≥15, respectively). (**b**) Western blot densitometry of H3K4me2 differences between high and low HDS samples. (**c**) Comparison of H3K4me2 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.

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 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). Results from Western blotting were used to quantify acetylome/phosphorylome differences between groups, and mass spectrometry was used to identify possible targets of BPS-causing PTM changes.

Results and discussion

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[48–50]. 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[51]. This effect is well described for some endocrine disruptors, and we suggest that it is also the case for BPS[52].

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[53]. Another candidate is outer dense fiber protein 2, for which insufficient phosphorylation leads to motility failure[54].

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[55] and activation of ATP synthases[56], suggesting their possible participation in BPS-caused motility decrease.

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.

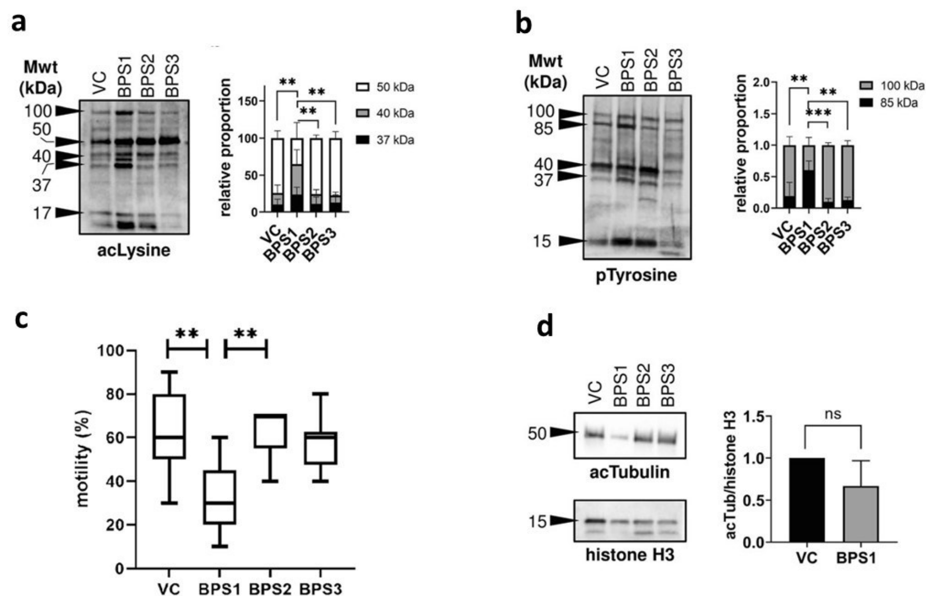

Figure 3. Effect of bisphenol S (BPS) on the sperm proteome and motility. (**a)** Representative Western blot of acetyl-lysine and densitometry analysis of major acetylated bands. Differences between groups treated with different BPS doses: BPS1 (0.001 µg/kg bw/day), BPS2 (1.0 µg/kg bw/day), BPS3 (100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

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). Redox PTMs play an irreplaceable role in redox balance maintenance, controlling protein activity and capacitation.

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 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.

Results and Discussion

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. 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[29]. 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)**.

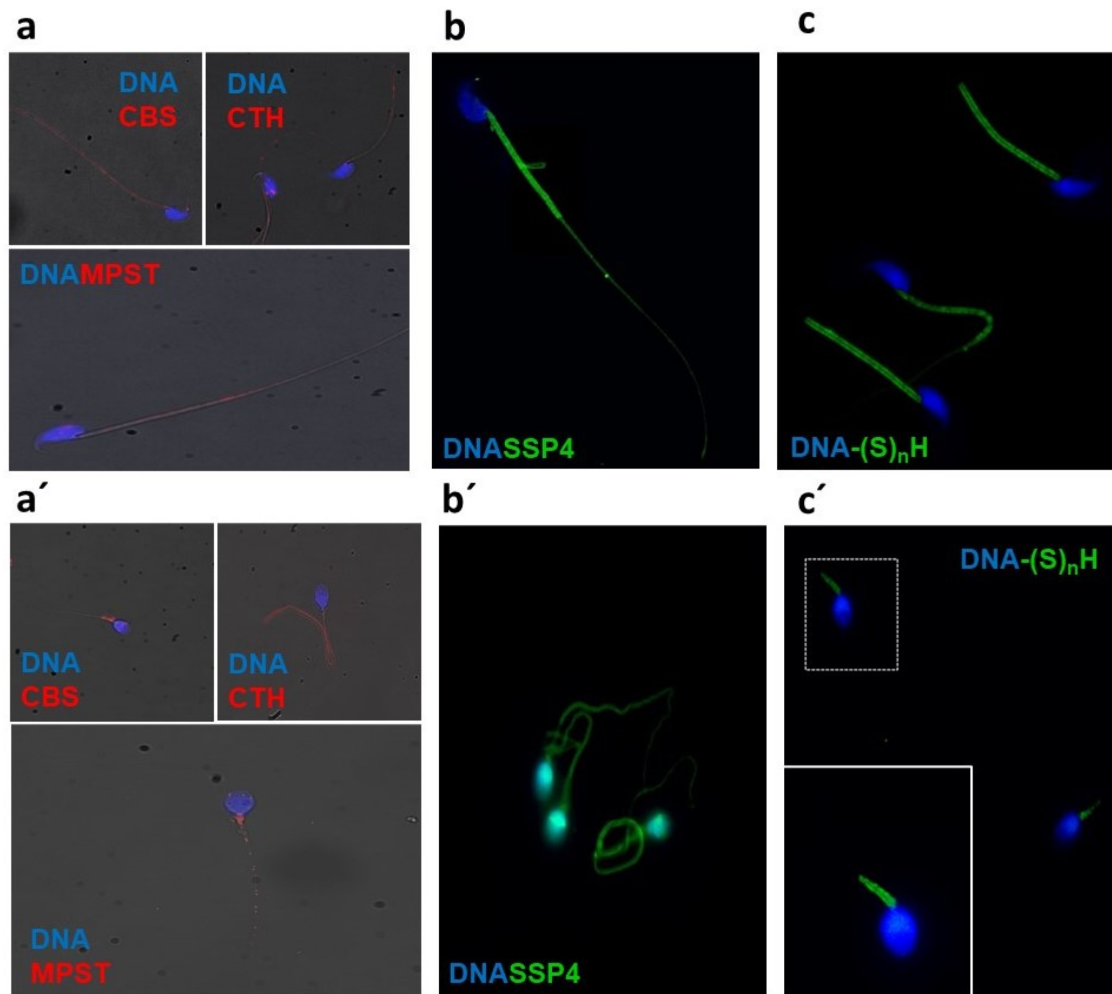

Figure 5. H_2S production and persulfidation (-$(S)_nH$) in **(a – c)** mouse and **(a´ – c´)** human spermatozoa. **(a, a´)** immunocytochemistry of cystathionine β-synthase (CBS), cystathionine γ-lyase (CTH) and 3-mercaptopyruvate sulfurtransferase (MPST). **(b, b´)** Localization of H_2S production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

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. Some of these proteins have been reported to undergo -$(S)_nH$, including glyceraldehyde-3-phosphate dehydrogenase, tubulin[60] and L-lactate dehydrogenase[58], 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[58], which supports the idea that S-nitrosylation serves as a -$(S)_nH$ precursor[61–63]. 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.

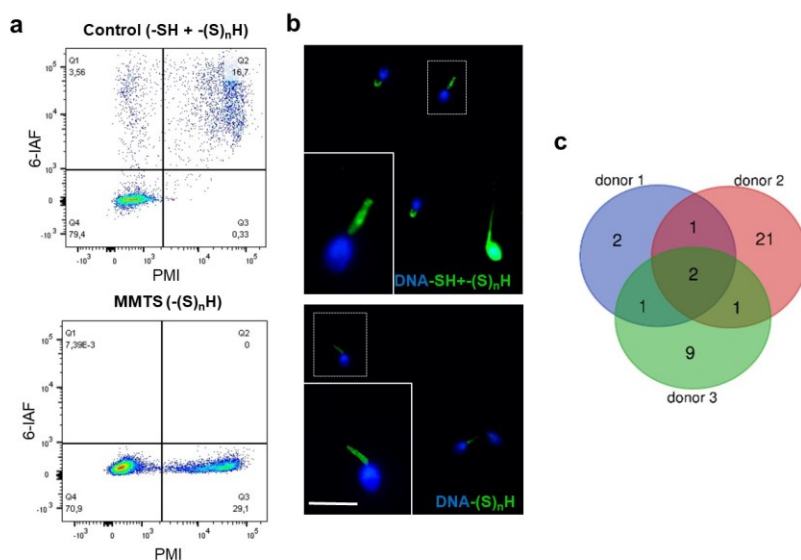

Figure 6. Free thiols and persulfidation analysis of proteins in human spermatozoa. (**a**) Flow cytometry of thiols (-SH) and persulfidation (-$(S)_nH$) in spermatozoa due to 6-iodoacetamidofluorescein (6-IAF) staining without (control) and with (MMTS) blocking of free thiols. The dot plot shows the separation of sperm subpopulations according to plasma membrane integrity (PMI) and 6-IAF signal intensity. (**b**) Representative images of -SH and -$(S)_nH$ detection *in situ* showing 6-IAF staining patterns in the MMTS and control groups. The white rectangle indicates the -$(S)_nH$-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.

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* incubationOur suggested H_2S dose is comparable to those previously used for spermatozoa treatment[27,28].

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. 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)**.

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[27,28,64], the mechanism of their action has remained unknown until now.

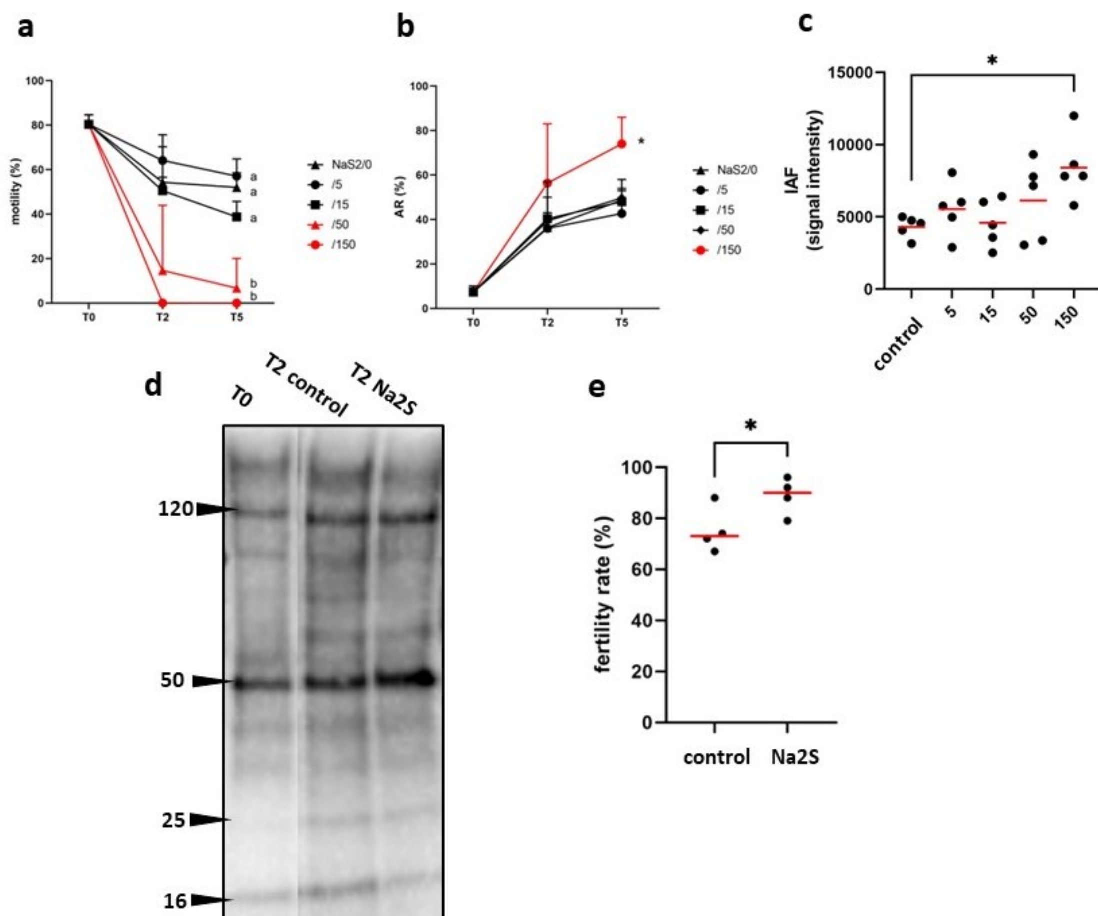

Figure 7. Spermatozoa treatment by different concentrations of H_2S donor (Na_2S). Test of the Na_2S concentration series, 0, 5, 15, 50, and 150 μM, on **(a)** sperm motility, **(b)** acrosome integrity, and **(c)** reduction ability over time. Concentrations with a harmful effect on spermatozoa are highlighted in red and considered to be toxic. **(d)** Protein persulfidation detection by WB according to treatment time and group. **(e)** Fertility rate differences between embryos raised from pretreated (Na_2S) and untreated (control) spermatozoa. 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.

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).

Řimnáčová H., Moravec J., Štiavnická M., Havránková J., Monsef L., Hošek P., Prokešová Š., Žalmanová T., Fenclová T., Petr J., Králíčková M., Nevoral J. 2022. Evidence of endogenously produced hydrogen sulfide (H_2S) and persulfidation in male reproduction. Submited to Scientific Reports.

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.

6. References

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7. APPENDIX

A1

Štiavnická M., García-Álvarez O., Ulčová-Gallová Z., Sutovsky P., Abril-Parreño L., Dolejšová M., Římnáčová 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**)

A2

Římnáčová 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**)

A3

Římnáčová H., Moravec J., Štiavnická M., Havránková J., Monsef L., Hošek P., Prokešová Š., Žalmanová T., Fenclová T., Petr J., Králíčková M., Nevoral J. 2022. Evidence of endogenously produced hydrogen sulfide (H_2S) and persulfidation in male reproduction. Submited to Scientific Reports.