Human sperm phosphoproteome reveals differential phosphoprotein signatures that regulate human sperm motility

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Running Title: Phosphoproteomic and human sperm motility

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ABSTRACT

Human sperm motility is essential for fertilization and among pathologies underlying male infertility is asthenozoospermia. Nevertheless, mechanisms regulating sperm motility are not completely unraveled. This work investigates phosphoproteins underlying human sperm motility by using differential phosphoproteomic in two human sperm subpopulations: high (HM) and low (LM) motility, obtained by centrifugation in a density gradient. Phosphoproteomics (HPLC-MS/MS triple TOF), comparing human LM and HM phosphoproteomes, identified 210 phosphopeptides with different abundance that correspond with 119 sperm proteins. Analysis showed that 40% of phosphoproteins in LM spermatozoa are involved in metabolism, (catabolism, protein transport, lipid biosynthesis), 25% in spermatogenesis and sperm function, 8% in immune system and 6% in DNA repair. In HM spermatozoa, 48% of phosphoproteins are related to spermatogenesis and sperm function (motility), whereas 8% are associated to metabolism. GSK3α resulted one of the most abundant phosphoproteins in HM spermatozoa. Western blot confirmed that GSK3α phosphorylation is higher in HM spermatozoa. Summarizing, this study i) identified phosphoproteins in two human spermatozoa populations, ii) supports that human spermatozoa rely in protein phosphorylation, such as GSK3 α, to regulate sperm motility, iv) raises the challenge of using some identified human sperm phosphorylated proteins (GSK3α) as targets to develop into clinically relevant biomarkers.

Keywords: Human spermatozoa, motility, phosphoproteomic, protein phosphorylation, glycogen shynthase kinase 3α

1. Introduction

Spermatozoon is a highly specialized haploid cell that functions with the only commitment to deliver paternal DNA to the oocyte. Due to this specialization, spermatozoa have lost most of their cytoplasm. Moreover, spermatozoa are characterized by containing a large quantity of protamine protein making sperm chromatin highly compacted [1]. As spermatozoa are not able to transcribe gene information into new synthetized proteins, these gametes regulate their cell functions mainly by post-translational modifications (PTM) of their present proteins, such as phosphorylation, acetylation, glycosylation, among others (reviewed by [2]). The PTM most extensively studied in spermatozoa by far is protein phosphorylation.

In the last 40 years, men seminogram parameters have fallen impacting negatively on sperm quality [3]. This is reflected in that 15% of couples worldwide in childbearing

age are having problems to conceive [4], where about 50% of fertility problems are associated to male factor [5]. Consequently, the use of assisted reproductive technologies (ART) has grown exponentially looking to bypass fertility problems [6, 7]. Interestingly, ejaculates contain a heterogeneous population of spermatozoa with different quality and responsiveness to stimulus [8, 9]. Motility is a special spermatozoa physiological feature that is activated after ejaculation and allows spermatozoa to move within the female reproductive tract. This characteristic fact makes nowadays the obtaining of high quality and motile spermatozoa, a crucial step for ART success. By using a density gradient, two different human sperm populations can be isolated with high or lower motility. This technique assures the selection of spermatozoa with higher motility associated to a better pregnancy achieved after performs ART [10].

The current standard descriptive semen analysis evaluation is an insufficient tool to provide appropriate diagnosis of the fertility potential, because even men within the reference range parameters established by the World Health Organization (WHO) may be unable to conceive [11]. Besides, it was shown that 30% of patients with normal semen analysis were associated with abnormal sperm function [12]. These facts emphasize that little is known about the mechanisms underlying molecular deregulation responsible for men infertility. In consequence, new methods are needed to discriminate better the sperm fertility potential.

Recently, several studies have used proteomic approaches in human spermatozoa reporting that distinct proteomic signatures distinguished high quality human spermatozoa from their low quality counterparts [13-16]. Therefore, the proteomic approach has recently been proposed as a tool to identify human sperm proteins as biomarkers of the fertility potential [15]. Given that sperm protein phosphorylation is specifically a key PTM that allows control of sperm physiology, the investigation the human phosphoproteome will undoubtedly contribute to unravel the control of main human sperm functions, but to date, only few studies have studied the phosphoproteome in human spermatozoa from healthy and asthenozoospermic donors. Thus, Ficarro et al. [17] using a 2D electrophoresis coupled to MS/MS analysis identified human sperm proteins phosphorylated, mainly in tyrosine residues, during capacitation. Chan et al. [18] using 2D electrophoresis MALDI-TOF MS established that 12 human sperm protein presented differential phosphorylation between normal and subfertile (with aberrant motility) spermatozoa. Later, Parte et al. [19] using Nano UPLC-MS^E tandem mass spectrometry identified 66 human sperm phosphoproteins differentially regulated

in severe asthenozoospermia with respect normozoospermic donors. Recently, Urizar-Arenaza et al. [20] using TMT labeling and LC-MS/MS identified sperm-specific protein changes downstream of kappa opioid receptor in human spermatozoa. However, none of these previous works have analyzed human sperm phosphoproteome in two human sperm populations that mainly differ in their motility (high and low). Therefore, the goal of this work is to investigate human sperm phosphoproteome using a different experimental approach: two human sperm populations differing in their motility degree in order to identify sperm phosphoproteins involved in the regulation of human sperm motility. This work will allow to i) identify protein phosphorylation sites that could potentially be used as prognosis tools to evaluate human sperm motility and/or quality and to ii) characterize the phosphorylation patterns of both human sperm subpopulations. Finally, these results could potentially lead to discriminate human spermatozoa fertility potential and they might also contribute to design novel strategies to bypass the health issue of men infertility.

2. Material and Methods

2.1. Chemicals and sources

The sperm density gradients were prepared with PureSperm® 100 and PureSperm® Buffer from Nidacon (Sweden, EU). Sperm Washing Medium (SWM) was from IrvineScientific (Daimler, St. Santa Ana, CA, USA). Propidium iodide (PI), SYBR-14 and MitoSOX™ Red probes were purchased from Molecular Probes (Leiden, The Netherlands); PNA-FITC was from Sigma- Aldrich (St Louis, MO, USA); JC-1 probe from Life Technologies Ltd (Grand Island, NY, USA); annexin-V–FITC from Immunostep (Salamanca, Spain); coulter isotone II diluent from Beckman Coulter Inc. (Brea, CA, USA); *DC* ™ and Bradford Protein Assays, and 2x Laemmli Sample Buffer from Bio-Rad (Hercules, CA, USA). ECL detection kit was from Thermo Scientific (Rockford, USA). Furthermore, the anti-α-tubulin, anti-phospho (Ser21/9) GSK3α/β, anti-GSK3α and anti-GSK3β polyclonal antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA).

2.2. Human semen samples

Samples from twelve healthy donors were obtained by masturbation into specific sterile containers after 2-4 days of sexual abstinence. Human sperm ejaculates were selected according to the recommendations of the World Health Organization, WHO [23] for normozoospermic subject. After complete liquefaction (between 10 minutes and 1 hour at 37 ºC with 5% of CO_2), samples were processed and the sperm parameters

(volume, sperm concentration and percentage of motility) were evaluated according to the recommendations of the WHO using a computer–assisted semen analyzer (CASA system). The average sperm count obtained by CASA in the 8 samples was 89 millions/mL, the average volume was 4.2 mL, the average total motility was 49%, the progressive motility was 39% and the rapid spermatozoa were 27%. The study was conducted in accordance with the ethical guidelines for human samples research and informed and written consent was obtained from all individual donors included in the study. The University of Extremadura Ethical Committee approved all protocols.

2.3. Human sperm fractions preparation

Two separated fractions of pure human spermatozoa differing in motility (low- and high-motility, referred here as LM and HM fraction respectively) were obtained by density gradient centrifugation using PureSperm as described by Netherton et al. [16] with some modifications. Briefly, aliquots of semen (100 millions/mL) were layered over the upper layer of the discontinuous density gradient 90%-60%-30% (v/v) using silane-coated silica particles and centrifuged at room temperature (RT) for 30 min at 500*g*. Following, the spermatozoa from the 90% pellet (HM) and the 90/60% interface (LM) were taken, washed once in SWM and centrifuged at RT for 10 min at 500*g*, then they were aliquoted out for further analysis.

2.4. Human sperm motility analysis

Sperm fractions (LM and HM) from 8 ejaculate samples were kept in SWM at 37 ºC in a 5% CO_2 incubator for 30 min prior evaluation of sperm motility. Each sample (6 µL) was placed in a pre-warmed Spermtrack sperm counting chamber coupled to the CASA system and the following motility parameters were evaluated using the ISAS® software (PROISER, Paterna, Valencia, Spain): percentages of motile, rapid and progressive spermatozoa. The settings of the CASA system were as follows: frame rate, 60 Hz; frame acquired, 25; rapid spermatozoa with average path velocity > 35 µm s^{-1}, progressive spermatozoa with a straightness threshold > 80%; temperature 37 °C. The minimum number of spermatozoa evaluated in each semen sample was at least 300.

2.5. Analysis of human sperm functional parameters by flow cytometry

Briefly, sperm fractions (LM and HM) for 8 ejaculate samples from 8 different donors were analyzed in duplicate. For each parameter 200,000 spermatozoa for each sperm fraction were incubated in SWM in darkness at RT with 20 nM of SYBR-14 probe and 9.6 µM of PI for 20 min to measure sperm viability (V); with 1.25 µg/ml of

PNA-FITC and 24 μM of PI for 5 min to measure acrosome membrane damage (AMD); with 2 μM of MitoSOX^{TM} for 15 min at 37 ºC and 5% CO_2 to measure mitochondrial anion superoxide production (SOP) and with 1.5 μM of JC-1 for 1 h at 37 ºC to evaluate mitochondrial membrane potential (MMP).

To study phosphatidylserine (PS) externalization to the outer leaflet plasma membrane 200,000 spermatozoa for each sperm fraction (LM and HM) were resuspended in the following buffer: 96 mmol/L NaCl, 4.7 mmol/L KCl, 0.4 mmol/L $MgSO_4$, 0.3 mmol/L NaH_2PO_4, 5.5 mmol/L glucose, 1 mmol/L sodium pyruvate, 21.6 mmol/L sodium lactate, 20 mmol/L HEPES (pH 7.45) and 2.5 mmol/L $CaCl_2$. The incubation with probes was performed adding 5 μL of annexin V-FITC per 1 million cells and 12 μM of PI for 15 min a RT.

For the analysis, a flow cytometer ACEA NovoCyte TM was used (ACEA Biosciences, Inc., San Diego, CA, USA) containing the ACEA NOVOEXPRESS TM software. The fluorescence values of SYBR-14, annexin V-FITC, JC-1 monomers and PNA-FITC were collected in the laser-excited fluorescence channel (BL1) using a 525-nm band-pass filter, whereas JC-1 aggregates and MitoSOX^{TM} fluorescence was collected in the BL2 channel using a 585-nm band-pass filter and PI fluorescence was collected in the BL3 channel using a 620-nm band-pass filter. The results were expressed as the average of the percentage of labeled spermatozoa for each parameter analyzed ± standard error of the mean (SEM). Sperm viability was considered as the percentage of SYBR-14^{+} and PI^{-} labeled cells. The SYBR-14 fluorescence spill-over into BL3 channel was compensated for (1%). Mitochondrial anion superoxide production was considered as the percentage of MitoSOX^{TM+}, BL1 overlapping signal on BL2 channel was compensated for (9,7%) and BL2 overlap signal on BL1 channel was compensated for (15%). PS externalization at the outer leaflet plasma membrane was evaluated using the percentage of annexin V^{+} and PI^{-} labeled spermatozoa. Acrosome membrane integrity was evaluated as the percentage of PNA-FITC^{+} and PI^{-} labeled spermatozoa. The percentage of orange-stained cells was recorded and considered the population of spermatozoa with a high mitochondrial membrane potential, BL1 spill-over the BL2 channel was compensated for (31.5%) and BL2 spill-over the BL1 channel was compensated for (13.2%). The results are expressed as the average of the orange-stained spermatozoa percentage ± SEM.

2.6. Protein extraction for proteomic analysis

As described by Netherton et al. [16], sperm LM and HM fractions were washed in phosphate buffered saline (PBS) and centrifuged at RT for 3 min at 10,000*g*, then were incubated in lysis buffer [7 M urea, 2 M thiourea, 1% (w/v) C7BzO (3-(4-heptyl)phenyl-3-hydroxypropyl)dimethylammonio-1-propanesulfonate), and 40 mM Tris (pH 10.4)] for 1 h at 4 °C with constant shaking. Supernatant was recovered after centrifugation at 18,000*g* for 15 min at 4 °C. Protein concentration was determined using the Bradford Protein Assay following the manufacturer' s instructions.

2.7. Western blot analysis

Briefly, samples from human sperm LM and HM fractions were washed in PBS for 3 min at 5,000*g* and then lysated in 2x Laemmli Sample Buffer for 10 min at 4 °C. The homogenates were clarified by centrifugation at 10,000*g* for 10 min at 4 °C and the supernatant, containing the sperm proteins solubilized was used for analysis of protein concentration. Protein concentration was determined using the *DC* ™ Protein Assay following the manufacturer' s instructions. For each sperm fraction, samples from a total of 8 human spermatozoa lysates were pooled in different combinations (n=3). Sperm proteins (10 μg) were resolved using 10% SDS-PAGE and electro-transferred to nitrocellulose membranes. Membranes were incubated at 4 °C overnight using anti-phospho-GSK3α/β, anti-GSK3α, anti-GSK3β (1:1,000) or anti-α-tubulin (1:5,000) polyclonal antibodies. After washing, membranes were incubated a RT for 45 min with the following secondary antibodies, anti rabbit IgG-HRP (1:10,000) or anti mouse IgG_K-HRP (1:5,000) and protein bands on the membrane were visualized using an ECL detection kit. Individual band volume intensities were quantified for each protein by densitometry on a gel documentation system using the ImageJ program to determine the relative intensity of HM vs. LM bands obtained in each pool for each antibody indicated.

2.8. Differential phosphoproteomics analysis by label-free quantification

For proteomic experiments, a total of 12 ejaculate samples from 12 donors were used. In order to achieve enough protein quantity, we have pooled 3 different ejaculate samples, resulting in 4 different ejaculate pools (n=4). Analysis was carried out following a bottom-up strategy for each fraction and using mass-spectrometry liquid chromatography (nano HPLC-MS/MS Triple TOF).

2.9. Protein precipitation and enzymatic digestion

Protein mixture was precipitated by the methanol/chloroform method, denatured in 7 M Urea/2M Thiourea/100 mM TEAB (pH 7.5) and reduced with 50 mM TCEP (pH 8.0) at 37 °C for 60 min. Cysteine residues were then alkylated with 200 mM methyl methanethiosulfonate (MMTS) for 10 min at RT. Urea/thiourea concentration was adjusted to 2 M in TEAB prior to sequence grade-modified trypsin addition in an enzyme-to-protein ratio 1:20 and samples were then incubated overnight at 37 °C. Resulting tryptic peptides were evaporated to dryness and stored at -20 °C for further analysis.

2.10. TiO_2 phosphopeptide enrichment

Peptide mixture was loaded onto titanium dioxide slurry, previously equilibrated in a highly acidic environment in the presence of glycolic acid (1M glycolic acid in 80% acetonitrile, 1% TFA), which has been shown to significantly improve selectivity by reducing unspecific binding of non-phosphorylated peptides, that is extremely useful in large-scale phosphoproteomics. The phosphopeptides bound to the TiO_2 resin are eluted from the chromatographic material using an alkaline buffer. Phosphopeptide enriched-fraction was subsequently desalted using an in-house Oligo R3 reversed-phase micro-column, dried and stored prior to be analyzed by tandem mass spectrometry. The unbound fraction containing non-phosphorylated peptides is saved.

2.11. Tandem mass spectrometry analysis of phosphorylated fractions

Phosphopeptide enriched-fractions were subjected to nano HPLC-MS/MS Triple TOF analysis using a nano liquid chromatography system (Eksigent Technologies nanoLC Ultra 1D plus, AB SCIEX, Foster City, CA) coupled to a high speed Triple TOF 5600 mass spectrometer (AB SCIEX, Foster City, CA) via a nanoelectrospray ion source. After injection, peptides were loaded onto a C18 PepMap trap column (5 µm, 100 µm I.D. x 2 cm, Thermo Scientific) working at 2 µL/min, in 0.1% formic acid in water. The trap column was switched on-line to a C18 nanoAcquity BEH analytical column (1.7 µm, 100 Å, 75 µm I.D. x15 cm, Waters). Equilibration was done in mobile phase A (0.1% formic acid in water), and peptide elution was achieved in a 120 min linear gradient from 5%-40% B (0.1% formic acid in acetonitrile) at 250 nL/min. The mass spectrometer was operated in data-dependent acquisition mode. For TOF scans, the accumulation time was set to 250 ms, and per cycle, up to 10 precursor ions were acquired.

2.12. Proteomics data analysis

MS/MS spectra were exported to mgf format using Peak View v1.2.0.3 and searched using Mascot Server 2.6.1, OMSSA 2.1.9, X!TANDEM Alanine 2017.2.1.4 and Myrimatch 2.2.140 against a composite target/decoy database built from sequences in the *Homo sapiens* reference proteome at Uniprot Knowledgebase (as of January 2018), together with commonly occurring contaminants. Search engines were configured to match potential peptide candidates with mass error tolerance of 15 ppm and fragment ion tolerance of 0.02 Da, allowing for up to two missed tryptic cleavage sites and a maximum isotope error (13C) of 1, considering fixed MMTS modification of cysteine and the following variable modifications: oxidation of methionine, phosphorylation of serine/threonine/tyrosine, possible pyroglutamic acid from glutamine or glutamic acid at the peptide N-terminus and acetylation of the protein N-terminus. Score distribution models were used to compute peptide-spectrum match p-values [21], and spectra recovered by a false discovery rate (FDR) <= 0.01 (peptide-level) filter were selected for quantitative analysis. Approximately, 15% of signals with the lowest quality were removed prior to further analysis. Differential regulation was measured using linear models [22], and statistical significance was measured using q-values (FDR). All analyses were conducted using software from Proteobotics (Madrid, Spain). Networks of protein-protein interactions among human phosphoproteins in sperm HM and LM populations were created using STRING version 11.0 (www.string-db.org).

2.13. Statistical Analysis

The program IBM SPSS 19 (Armonk, NY, USA) has been used to perform the statistical analysis. The mean and SEM were calculated for descriptive statistics. Data were tested for normal distribution with a Kolmogorov-Smirnov test and for homoscedasticity with a Levene test. Differences in motility parameters by CASA data and sperm functional parameters by flow cytometry data between the both human sperm fractions (HM and LM) were determined by a parametric t test (Student´s *t*-test). Results were considered significant when p values were lower than 0.05.

3. Results

3.1. Motility data by CASA confirm differences between low (LM) and high (HM) motility human fractions

To characterize each fraction, sperm motility parameters were analyzed by CASA The results shown that the average total motility was 42.1%, the progressive motility was 27.5% and the rapid progressive spermatozoa were 19.8% in the LM fraction, while

these percentages were significantly higher (90.0%, 79.1% and 58.4%, respectively) in spermatozoa from HM fraction (Figure 1).

In view of the percentages of the motility parameters shown by LM spermatozoa, this fraction presents characteristics of the asthenozoospermic samples (progressive motility < 32%) according to WHO [23].

3.2. Flow cytometry data reveal different sperm qualities between LM and HM human fractions

In order to determine other possible functional differences, besides motility, between LM and HM fractions, we analysed important sperm functional parameters by flow cytometry. As shown in Figure 2, results in HM sperm fraction show higher sperm viability (87.6% vs. 78.1% in LM) and high MMP (45.3% vs. 18.7% in LM), whereas results in LM fraction have increased superoxide anion production (27.5% vs. 15.8% in HM) and outer phosphatidylserine exposure in spermatozoa (6.0% vs. 1.2% in HM). No significant differences in spermatozoa with the acrosome membrane damaged (0.9% vs 0.2% in HM) were observed between both human sperm fractions (Figure 2B).

These flow cytometry results, together with those observed in motility (Figure 1), confirm clear functional differences between both LM and HM human fractions in our experimental conditions.

3.3. Differential phosphopeptides abundance between LM and HM human spermatozoa

The phosphoproteomic analysis comparing the human phosphoproteomes of LM and HM sperm fractions resulted to be a very rich experiment in phosphorylated species with a high number of identified and quantified phosphopeptides. Therefore, 380 phosphopeptides with a differential abundance were detected considering a q-value < 0.05 (good probability), and up to 217 phosphopeptides if we use a q-value more restrictive, < 0.01 (high confidence). In view of these results we decided to work with a q-value < 0.01 of high confidence, as the expected proportion of false positives under this condition does not exceed 1%. In addition, 7 of the 217 phosphopeptides differentially abundant were associated to more than one sperm protein, so finally we decided to focus on the 210 phosphopeptides linked to a single protein, which render a total of 119 phosphoproteins with different abundance between low and high motility human sperm fractions (Table 1). Considering the 210 phosphopeptides differentially detected, 102 out of them were more abundant in the HM human sperm fraction, whereas 108 were more abundant in the LM human sperm fraction. Some of these

phosphopeptides are showed in Table 2 while the whole list of differentially abundant human sperm phosphopeptides is reported in Table S1 in the Supplementary Data.

When we focus in the low motility sperm fraction, the phosphopeptide with the highest relative abundance corresponds with the heat shock protein beta-1 (HSPB1, 24 times higher in LM vs. HM fraction, see Table 2), which was also the phosphopeptide with the most differential abundance between both fractions. On the other hand, the phosphopeptide with the lowest relative abundance (1.7 times) in the LM fraction corresponds with PH and SEC7 domain-containing protein 3 (PSD3, Table 2). Regarding the 102 phosphopeptides most abundant in the high motility human sperm fraction, the phosphopeptide with the highest (16 times higher amount in HM vs. LM fraction) and the lowest (1.5 times) relative abundance corresponds to fibrous sheath-interacting protein 2 (FSIP2) together with FAM186A protein (Table 2).

3.4. Distribution of the phosphosites in the human sperm phosphopeptides

Among the phosphorylation sites identified, the analysis of the distribution of phosphorylated amino acids shows that the proportion of phosphoserine, phosphothreonine, and phosphotyrosine occupied 92%, 8%, and 0% of the total sites, respectively (Figure 3). Our results also found novel phosphorylation sites in proteins from human spermatozoa. In fact, more than 60% of the phosphosites identified (131 out of 210) have not been previously identified in human cells, according to PhosphoSitePlus database (https://www.phosphosite.org) (Undescribed phosphosites, Table 1).

3.4. Differential phosphoproteins between LM and HM human sperm fractions

As mentioned before, the comparison between the phosphoproteins of high and low motility sperm fractions results in the identification of 210 phosphopeptides with different relative abundance. Because many sperm proteins were modified by phosphorylation at multiple sites, these 210 phosphopeptides actually corresponded with 119 human sperm proteins, 67 out of them are significantly more abundant in LM, whilst 48 proteins are significantly more abundant in HM spermatozoa. Phosphoproteomic results also found 4 proteins that have phosphopeptides present in both fractions, two of them are related with reproductive process as sperm-egg recognition and spermatogenesis, one with metabolism and the function of the last one is unknown. The complete list of human sperm proteins with different relative abundance between LM and HM fractions is reported in Table S2 in the Supplementary Data.

The distribution of the 119 identified proteins according to their biological function is shown in Figure 4 where human sperm proteins were classified according to their main function using the information available at Reactome (http://www.reactome.org) and at the UniProtKB/Swiss-Prot (https://www.uniprot.org) databases. The most abundant group corresponds to proteins involved in spermatogenesis and sperm function (35%), followed by proteins involved in metabolism (27%). Sperm proteins with uncharacterized or unknown function and those with other functions represent each group 19% of phosphoproteins with differential abundance between both LM and HM fractions.

Regarding LM sperm fraction, its low motility and quality characteristics seem to be associated with a higher abundance of phosphoproteins involved in metabolism cellular, as 40% of the most abundant phosphopeptides in LM spermatozoa belong to proteins involved in metabolism (Figure 5A). In particular, these sperm proteins are related to regulation of catabolic processes, protein transport or lipid biosynthesis. The other groups of phosphoproteins more abundant in LM fraction are involved in spermatogenesis and sperm function (25%), immune system (8%) and DNA repair (6%), while those phosphoproteins with other functions are 13% and uncharacterized or unknown represent 8%. Regarding HM fraction, spermatozoa exhibiting high motility and quality parameters are associated with a much lower presence of phosphoproteins involved in sperm metabolism (8%) than those from the LM fraction (Figure 5B). The predominant protein function in high motility and quality spermatozoa (48%) is related to proteins involved in spermatogenesis and sperm function, as motility (Figure 5B), whereas 36% of proteins possess uncharacterized or unknown function and 8% are included in other functions.

3.5. GSK3α is one of the most abundant phosphoproteins in HM human spermatozoa

Phosphoproteomic results have allowed us to identify glycogen synthase kinase 3 (GSK3) as one of the most abundant phosphoproteins in human spermatozoa exhibiting high motility and quality. Thus, as seen in Table 3, the abundance of its isoform GSK3α is at least twice greater in HM fraction than in LM sperm fraction (column log2 fold change HM/LM ~ 1.1). Moreover, there are up to five phosphorylation sites of GSK3α protein detected and two of them (Ser20 and Ser21) show the log2 fold change HM/LM of 0.92 and ~ 0.85, respectively, which indicate that the relative abundance of phospho-Ser21 GSK3α correlates with the amount of GSK3α protein. One of the phosphosites, the one corresponding to the phosphorylated Ser21 residue, has been associated recently

with low GSK3 catalytic activity and high motility in human spermatozoa [24]. In view of these findings, we decided to further validate GSK3α phosphoproteomic result by Western blot analysis using specific antibodies against GSK3α and phospho-Ser21 GSK3α in sperm lysates from both, LM and HM human sperm fractions. As seen in Figure 6, GSK3α levels (upper film) are lower in LM fraction compared to HM human sperm fraction. Similarly, the amount of phospho-Ser21 GSK3α is much lower in spermatozoa with low motility than those with high motility (middle film). These data confirm the phosphoproteomics results about GSK3α and also validate this label-free quantitative approach to phosphoprotein analysis performed in human spermatozoa. Moreover, phophoproteomics analysis does not detect differences in GSK3β between human HM and LM spermatozoa and the Western blot results about this isoform were not consistent.

4. Discussion

A routine assisted reproductive technology (ART) is the sperm selection by centrifugation using a density gradient that assures the selection of spermatozoa with the best quality parameters, related to a better pregnancy following ART such as IVF or ICSI. Moreover, the elucidation of sperm proteins that regulate human sperm motility is an essential discovering that needs to be addressed. This study, using phosphoproteomic analysis, reveals important differences in the human sperm phosphoproteome according to the sperm motility degree (low versus high motility). Importantly, our results clearly confirm functional and quality differences between two human sperm populations isolated in this study, LM and HM. Thus, the low motility fraction of human spermatozoa (42% of average total motility, 28% of progressive motility and 20% of rapid progressive spermatozoa) exhibits concomitantly much lower viability, lower mitochondrial membrane potential, as well as higher superoxide anion production and higher outer phosphatidylserine translocation than the HM spermatozoa. This later human sperm population (90% of average total motility, 79% of progressive motility and 58% of rapid progressive spermatozoa) possesses higher viability and mitochondrial membrane potential as well as lower superoxide anion production and lower phosphatidylserine translocation than the LM sperm population. These results not only confirm a clear distinction of both human sperm fractions, LM and HM, based on their motility and quality, but more importantly, they also validate our human sperm fractionation technique to later approach a phosphoproteomic study.

Phosphoproteomic analysis reveals that 210 phosphopeptides belonging to a total of 119 proteins of human spermatozoa are detected at differential amounts according to their motility degree. Among them, 67 sperm phosphoproteins are more abundant in the LM sperm population, whereas 48 phosphoproteins are predominant in the HM population. To our knowledge, this is the first work studying the phosphoproteome of human sperm proteins in two sperm populations isolated by their different motility. However, there are few previous studies analyzing phosphoproteome in human spermatozoa from healthy and asthenozoospermic donors. Thus, previously, Chan et al. [18], using 2D electrophoresis MALDI-TOF MS, found only 12 human sperm proteins with differential phosphorylation between normal and subfertile donors, whereas later Parte et al. [19] using Nano UPLC-MS^E tandem mass spectrometry, detected 66 human sperm phosphoproteins significantly different in asthenozoospermic with respect normal donors. Differences in the number of phosphoproteins detected may be due to i) the distinct phosphoproteomic approach used in each study and also ii) to the differences between initial human sperm samples: with aberrant motility [18], severe asthenozoospermia [19] and LM and HM fractions isolated from healthy donors (present study).

Analysis of phosphorylated amino acids distribution in human spermatozoa identified by our phosphoproteomic shows that the proportion of phosphoserine, phosphothreonine, and phosphotyrosine (92%, 8%, and 0%, respectively) is consistent with findings in vertebrate cells showing that the most frequent protein phosphorylation occurs at serine, being the relative frequency of serine, threonine and tyrosine phosphorylation being in an approximate ratio of 1,000:100:1 [25]. Ficarro et al. [17], investigating the phosphoproteome in capacitated human sperm by tandem mass spectrometry (MS/MS), found several proteins that were phosphorylated in tyrosine. However, we have not detected phosphorylation in tyrosine residues, which could be explained by the fact that we isolated both sperm fractions under non-capacitating conditions.

Interestingly, this study shows that phosphorylated proteins of human spermatozoa exhibiting HM are predominantly involved in spermatogenesis and sperm function, such as motility. Both sperm processes are essential contributors to the fertilization potential of human spermatozoa and therefore to the reproductive success. This is consistent with the fact that HM human spermatozoa also exhibit the best quality parameters. Previously, Parte et al. [19] found that deregulated phosphoproteins

between normal and asthenozoospermic human spermatozoa included predominantly heat shock proteins (HSPs), cytoskeletal and are also associated to the fibrous sheath proteins and those related to energy metabolism. Thus, our findings are in agreement with this work, suggesting a differential regulation mediated by phosphorylation of human sperm proteins that control motility. In particularly the differentially regulated phosphoproteins are mainly involved in key requirements of the flagellum necessary for the sperm movement, such as maintenance of intracellular high energy levels, proteins mediating folding and preventing protein aggregation, and also proteins controlling axoneme mechanical components such as proteins involved in the fibrous sheath and cytoskeleton [19]. In fact, we have detected several phosphoproteins more abundant in the human HM spermatozoa that exert functions associated to flagellum assembly and motility, such as the fibrous sheath-interacting protein 2 FSIP2, the outer dense fiber protein ODF3B, proteins containing coiled-coil domain (CCD): CCD63, CCD151, CCD183, CCD188, CCD38 also proteins associated to cilia and flagella: CFA77, CFA91, PCDP1. Also, we detected heat shock proteins such as HSPB1 and HSP90A that are differentially phosphorylated between HM and LM fractions, in agreement with other HSPs found by Parte et al. [19] and supporting the role of HSPs in human sperm function and male fertility.

On the other hand, the phosphorylated proteins abundant in LM human spermatozoa are mainly involved in sperm metabolism, such as FAD synthase FAD1, and particularly related to regulation of catabolic processes, protein transport or lipid biosynthesis. These results are in agreement with those from the previous phosphoproteomic study by Parte et al. [19], which found carbohydrate metabolic pathways altered in asthenozoosperm such as those of inositol, fructose, mannose and gluconeogenesis. In fact, we have detected more phosphorylated abundance of pyruvate dehydrogenase E1 component subunit alpha ODPA, mannose-6-phosphate isomerase MPI or ATP-citrate synthase ACLY in the LM human sperm fraction. Moreover, our phosphoproteomic analysis also identified more abundance of proteins involved in lipid metabolism as dihydroxyacetone phosphate acyltransferase GNPAT, ethanolamine-phosphate cytidylyltransferase PCY2 or choline-phosphate cytidylyltransferase B, PCY1B.

Moreover, the present study also identified human sperm phosphoproteins with uncharacterized or unknown functions that represent almost 20% and also has identified novel protein phosphorylation sites. Therefore, the challenge of elucidation of these

sperm phosphoproteins functions as well as investigation of novel phosphorylation sites will be undoubtedly of great interest for unraveling the molecular mechanisms based on protein phosphorylation underlying different motility patterns in human spermatozoa.

Interestingly, one of the human sperm phosphoproteins identified as more abundant in human sperm HM fraction is the α isoform of glycogen synthase kinase 3, GSK3, a serine/threonine kinase that contains at least 5 phosphorylation sites in the following serine positions 2, 7, 14, 20 and 21. In fact, it has been demonstrated that the sperm GSK3α phosphorylation at Ser21 regulates its kinase activity, which negatively regulates motility in bovine [26], porcine [27], murine [28] and caprine spermatozoa [29]. It has been demonstrated that spermatozoa are unique in their specific requirement of GSK3α for normal motility and male fertility [30]. More recently, the regulatory role of GSK3α in motility has also been found in human spermatozoa as the activity of specific GSK3α isoform (but no GSK3β) is negatively correlated with human sperm motility [24]. Therefore, we aimed to further confirm phosphoproteomic analysis by specifically studying GSK3α phosphorylation at Ser21 in ejaculated human spermatozoa exhibiting both low and high motility. The amount of phospho-Ser21 GSK3α detected by Western blot is significantly higher in human spermatozoa presenting HM in total coincidence with phosphoproteomics data. Therefore, these results firmly validate this label-free quantitative approach to phosphoproteins analysis performed in human spermatozoa. Moreover, our results in human sperm populations isolated by their different motility, are in agreement with the recent study by Freitas et al. [24] reporting that total GSK3α and phospho-Ser21 GSK3α levels are lower in human asthenozoospermic samples compared to normospermic. Moreover, the same study found that GSK3α is required for human progressive sperm motility, which is in line with our data showing higher abundance of phospho-Ser21 GSK3α in HM spermatozoa exhibiting higher progressive motility. In addition, Freitas et al. [24] demonstrates that while GSK3α phosphorylation is a negative modulator, GSK3β appears does not influence human sperm motility. These authors localized the majority of GSK3α mainly in the sperm tail, whereas GSK3β is present on the sperm head and proposed that this subcellular distribution of GSK3 isoforms leads to different interactions with other proteins that could explain the distinct role of each GSK3 isoform in human sperm motility [24].

5. Conclusions

In conclusion, phosphoproteomics by nano HPLC-MS/MS Triple TOF, revealing the differentially abundance of human sperm phosphoproteins in low sperm motility versus high sperm motility populations, has identified human sperm proteins subjected to PTM by phosphorylation that are directly involved in the regulation of human sperm motility, such as GSK3α. Therefore, this study provides further evidence to support that mature human sperm cell relies on protein PTM to regulate sperm motility, specifically protein phosphorylation. These results acquire high relevance in the ART field based on two facts. i) First, a common cause of human male infertility is due to asthenozoospermia that is characterized by decreased sperm motility (< 40%) or reduced progressive motility (< 32%) according to WHO [23]. Moreover, proteins that physiologically regulate sperm motility are rather unknown and therefore the molecular mechanisms of asthenozoospermia are not yet elucidated. ii) Second, an important concern in AR is related to is effectiveness, as only ~ 25–40% of all treatment cycles are successful, and to date no markers are available to predict whether a treatment will be effective. Thus, some of the human sperm phosphorylated proteins identified in this study, as GSK3α, whose phosphorylation plays a key role in the regulation of sperm motility in several species [26-29] and importantly also in human spermatozoa [24], could be considered as potential biological targets for diagnostic and prognostic biomarkers in the clinic or ART.

Supplementary information is linked to the online version of the paper on the Journal of Proteomics website.

Funding sources

This work was supported by the regional Grants from Junta de Extremadura (Spain) IBI16184 and GR18094. David Martin-Hidalgo is recipient of a post-doctoral fellowship from Junta de Extremadura and European Social Funds (PO17020).

Acknowledgements

We are very thankful to donors and also Beatriz Macias-Garcia and Violeta Calle-Guisado for their kind and excellent help obtaining human semen samples and Lauro Gonzalez-Fernandez for his always-kind support in the laboratory. We also thank the staff of the Biotechnology National Center, CNB in Madrid (Spain) that belongs to ProteoRed, PRB-3-ISCIII, where the proteomics analysis was performed, and we especially acknowledge Rosana Navajas for her excellent technical support to during preliminary experiments performed to optimize the human sperm lysates preparation before the phosphoproteomics analysis. We thank Proteobotics S. L, Madrid (Spain)

and especially to Antonio Ramos for his excellent assistance in the phosphoproteomic data analysis. David Martin-Hidalgo is recipient of a post-doctoral fellowship from Junta de Extremadura (Spain) and European Social Funds (PO17020). The research group SINTREP belongs to "Excellence network in the *in vitro* production of animals with veterinarian interest" (Ref. AGL2016- 81890-REDT from the Ministerio de Ciencia, Innovación y Universidades (Spain).

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Figure legends

Figure 1. Main human sperm motility parameters in low (LM, white histograms) and high (HM, black histograms) motility fractions obtained by density gradient. Results are expressed as percentage of total spermatozoa analyzed. Data are expressed as mean ± SEM; ** $P < 0.01$; (n=8).

Figure 2. Functional parameters indicative of human sperm quality in low and high motility fractions. Sperm viability (V), mitochondrial superoxide anion production (SOP) and high mitochondrial membrane potential (hMMP) in A and outer phosphatidylserine translocation (PS) and acrosome membrane damage (AMD) in B, were analyzed in low (LM, white histograms) and high motility (HM, black histograms) fractions of human spermatozoa. Data, expressed as percentage of total spermatozoa analyzed, are shown as mean ± SEM; *$P < 0.05$; ** $P < 0.01$; (n=8).

Figure 3. Distribution of all quantified phosphorylated serine, threonine and tyrosine residues in the 119 human sperm phosphoproteins differentially abundant in our study using nano HPLC-MS/MS Triple TOF analysis. q-value < 0.01

Figure 4. Distributions of biological functions of the 119 human sperm proteins containing phosphopeptides with differential abundance between human HM and LM fractions. This graph was performed according to the information available at the UniProtKB/Swiss-Prot (https://www.uniprot.org) and Reactome (http://www.reactome.org) Web sites.

Figure 5. Distribution of human sperm proteins according to the biological function of the proteins with phosphopeptides detected more abundant in LM and in HM sperm fraction (A and B, respectively). This graph was performed according to the information available at the UniProtKB/Swiss-Prot (https://www.unirprot.org) and Reactome (http://www.reactome.org) Web sites.

Figure 6. Relative abundance of GSK3α and phospho-Ser21 GSK3α (P-GSK3α) in human low motility (LM) and high motility (HM) spermatozoa. Samples from HM and LM fractions from a total of 8 human spermatozoa lysates were pooled in three different

combinations (n=3). Then, protein lysates (10 µg) were analyzed by western blotting using anti-GSK3α and anti- phospho GSK3α/β specific antibodies. Protein loading controls were performed using anti-α-Tubulin antibody. Lower graphs depict the amounts of GSK3α (left) and P-GSK3α (right) quantified by densitometry and normalized to the amount of α-tubulin. Values are expressed as mean ± SEM (n=3)

Table legends

Table 1. Phosphorylation differences between human HM and LM spermatozoa detected by label-free quantitative phosphoproteomics nano HPLC-MS/MS Triple TOF analysis. The numbers of differential abundance of phosphopeptides, the corresponding proteins and the new identified phosphosites are shown. q-value < 0.01.

Table 2. List of some phosphopeptides detected at amounts statistically different between human low (LM) and high motility (HM) sperm fractions and their corresponding phosphoprotein names. [a,b] Results are expressed as log2 ratios and antilog2 ratios of phosphopeptides levels of human HM referred to LM sperm fractions. The positive or negative sign for log2 ratios depends on whether the phosphopeptide is more abundant in HM or in LM fraction, respectively.

Table 3. Differential abundance of phosphopeptides of GSK3α protein detected in human LM and HM sperm fractions by label-free quantitative phosphoproteomics nano HPLC-MS/MS Triple TOF analysis. Phosphorylation sites of this protein are indicated (phosphosite) as well as the peptide sequence identified (peptide). [a,b] Results are expressed as log2 ratios and antilog2 ratios of phosphopeptides levels of HM referred to LM human sperm fractions. The positive sign for log2 ratios shows whether the phosphopeptide is more abundant in HM.

Table 1. Phosphorylation differences between human HM and LM spermatozoa detected by label-free quantitative phosphoproteomics nano HPLC-MS/MS Triple TOF analysis.

	TOTAL
Phosphopeptides	210
Phosphoproteins	119
Undescribed phosphosites	131

The numbers of differential abundance of phosphopeptides, the corresponding proteins and the new identified phosphosites are shown. q-value < 0.01

Table 2. List of some phosphopeptides detected at amounts statistically different between human low (LM) and high motility (HM) sperm fractions and their corresponding phosphoprotein names.

Protein name	Phosphopeptide	Log2 (HM/LM)[a]	Antilog2 (HM/LM)[b]	p value	q value
FSIP2	NYSLGSPD...	4.019	16.212	3.00E-03	0.002
ODF3B	TSLPQDNTR	2.822	7.071	3.00E-03	0.002
CQ047	...TLASHASSR	2.656	6.303	6.00E-03	0.003
EFCB5	RMSAAEQG...	2.649	6.272	0.001	0.003
TAU	IGSLDNITHVP...	2.518	5.728	0.001	0.003
CC183	EASFIEER	2.492	5.626	3.00E-03	0.003
CKLF1	SAQSAAAARP...	2.372	5.177	0.001	0.003
CCD63	GDSLPEKVDD...	1.905	3.745	0.001	0.003
CC151	AASANALPPQ...	1.876	3.671	0.001	0.003
S31D1	ASTSNETEIFPP	1.847	3.598	0.001	0.003
ADGB	ATSQGNTASQ...	1.316	2.490	0.005	0.005
F205A	QPGSASALGYP	1.242	2.365	0.001	0.003
CFA91	...QAQPQVSQTR	0.804	1.746	0.005	0.006
S31E1	ASSGSVQED...	0.776	1.713	0.005	0.006
CASC1	AISKEVEEE...	0.749	1.680	0.006	0.007
S26A8	ETYSETDKN...	0.721	1.649	0.006	0.007
PCDP1	QDSTTQLSGK	0.694	1.617	0.006	0.007
EIF3B	SDSRAQAVS...	0.680	1.602	0.008	0.008
FAM186A	VLPGPSPQS...	0.598	1.513	0.011	0.010
HSPB1	QLSSGVSEIR	-4.569	23.736	3.00E-03	0.001
ZCH18	LGVSVSPSR	-4.096	17.101	3.00E-03	0.002
HS90A	DKEVSDDEAE...	-2.087	4.249	0.001	0.003
PGRC1	EGEEPTVYSD...	-1.935	3.824	0.005	0.006
ACLY	TASFSESR	-1.727	3.310	0.001	0.003
UBR4	HVTLPSSPR	-1.314	2.486	0.004	0.006
EMAL4	APVSSTESVIQ...	-1.189	2.280	0.005	0.006
PSB6	DGSSGGVIR	-1.124	2.180	0.005	0.006
DNAI1	...YRDELVAGS...	-1.113	2.163	0.004	0.006
SCAM2	AASSAAQGAF...	-1.041	2.058	0.004	0.005
DCTN1	QSQIQVFEDG...	-1.011	2.015	0.005	0.006
Q16531	... ALSSSVSSSK	-0.968	1.956	0.006	0.007
ATG9A	RESDESGESA...	-0.877	1.837	0.007	0.008
MPI	MGSNSEVAR	-0.845	1.796	0.011	0.010
GNPAT	SSYNLVPR	-0.804	1.746	0.008	0.008
KLH10	YSASTSTLPV	-0.786	1.724	0.01	0.009
TEX50	VATTASVIYK	-0.757	1.69	0.009	0.009
FSCN3	YGYVGSSSGH...	-0.742	1.672	0.011	0.010
PSD3	SHSSPSLNPDT...	-0.728	1.656	0.012	0.010

[a,b]Results are expressed as log2 ratios and antilog2 ratios of phosphopeptides levels of HM referred to LM human sperm fractions. The positive or negative sign for log2 ratios depends on whether the phosphopeptide is more abundant in HM or in LM, respectively.

Table 3. Differential abundance of phosphopeptides of GSK3α protein detected in human LM and HM sperm fractions by label-free quantitative phosphoproteomics nano HPLC-MS/MS Triple TOF analysis.

Protein name	Phosphosite	Peptide	Log2 (HM/LM)[a]	Antilog2 (HM/LM)[b]	p value	q value
GSK3α	----	---	1.111	2.160	0.001	0.003
	S20-p	T<u>S</u>SFAEPG…	0.919	1.891	0.006	0.007
	S21-p	TS<u>S</u>FAEPG…	0.848	1.800	0.014	0.011
	S14-p	GGGPGG<u>S</u>G...	0.528	1.442	0.028	0.019
	S2-p	<u>S</u>GGGPSGG...	0.470	1.385	0.033	0.022
	S7-p	SGGGP<u>S</u>GG…	0.397	1.317	0.078	0.044

Phosphorylation sites of GSK3α are indicated (phosphosite) as well as the peptide sequence identified (peptide). [a,b] Results are expressed as log2 ratios and antilog2 ratios of phosphopeptides levels of HM referred to LM human sperm fractions. The positive sign for log2 ratios shows whether the phosphopeptide is more abundant in HM.

Figure 1

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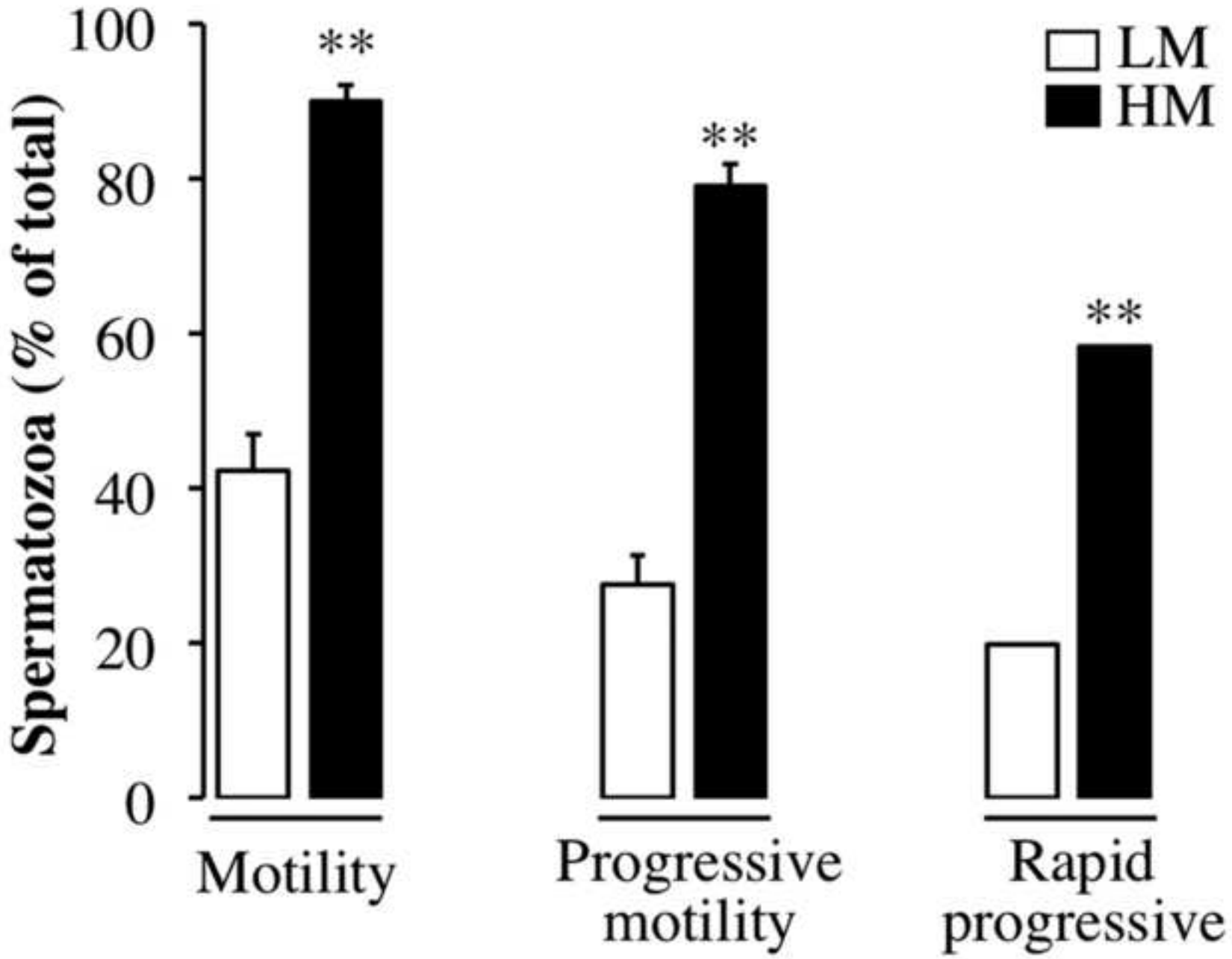

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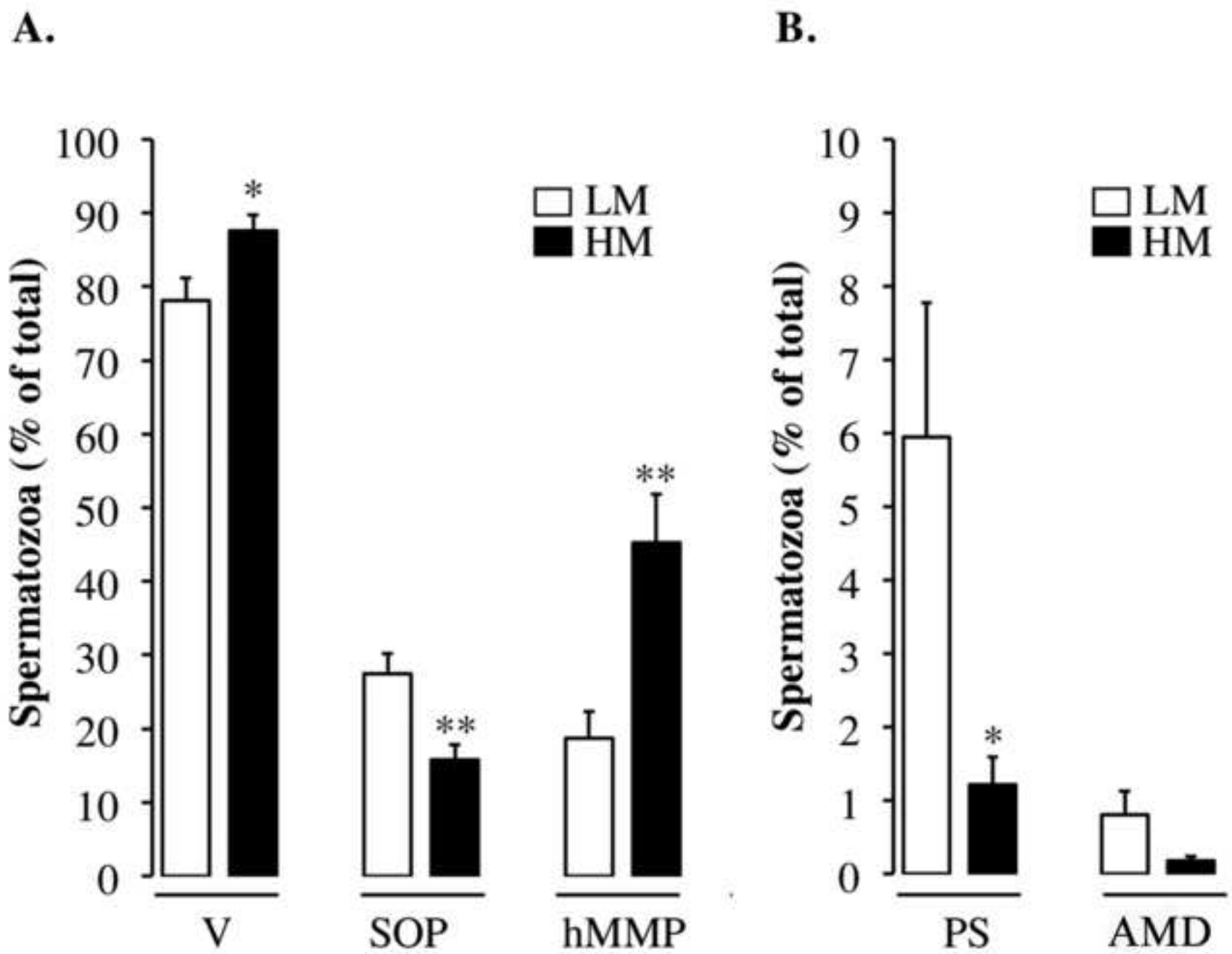

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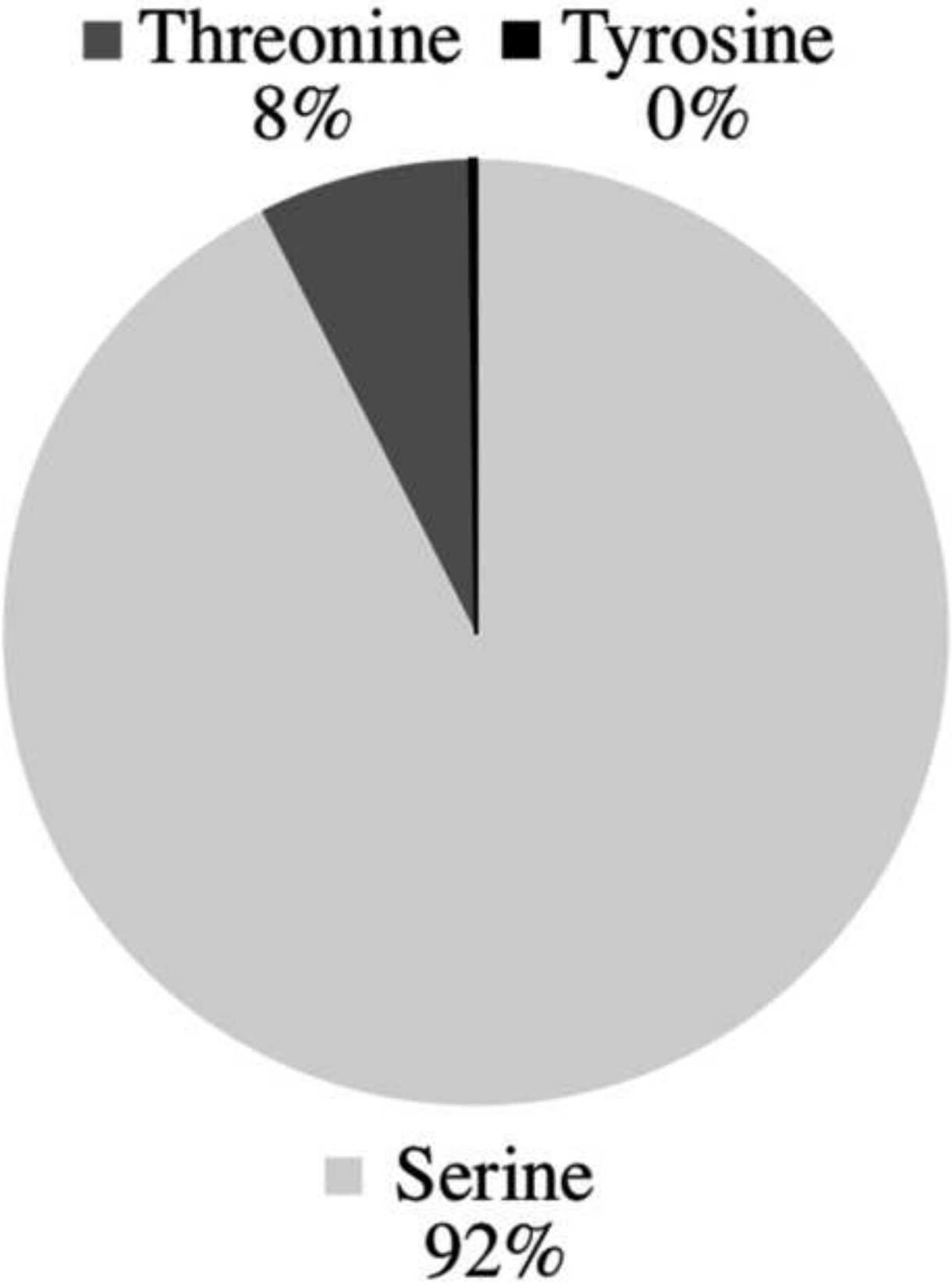

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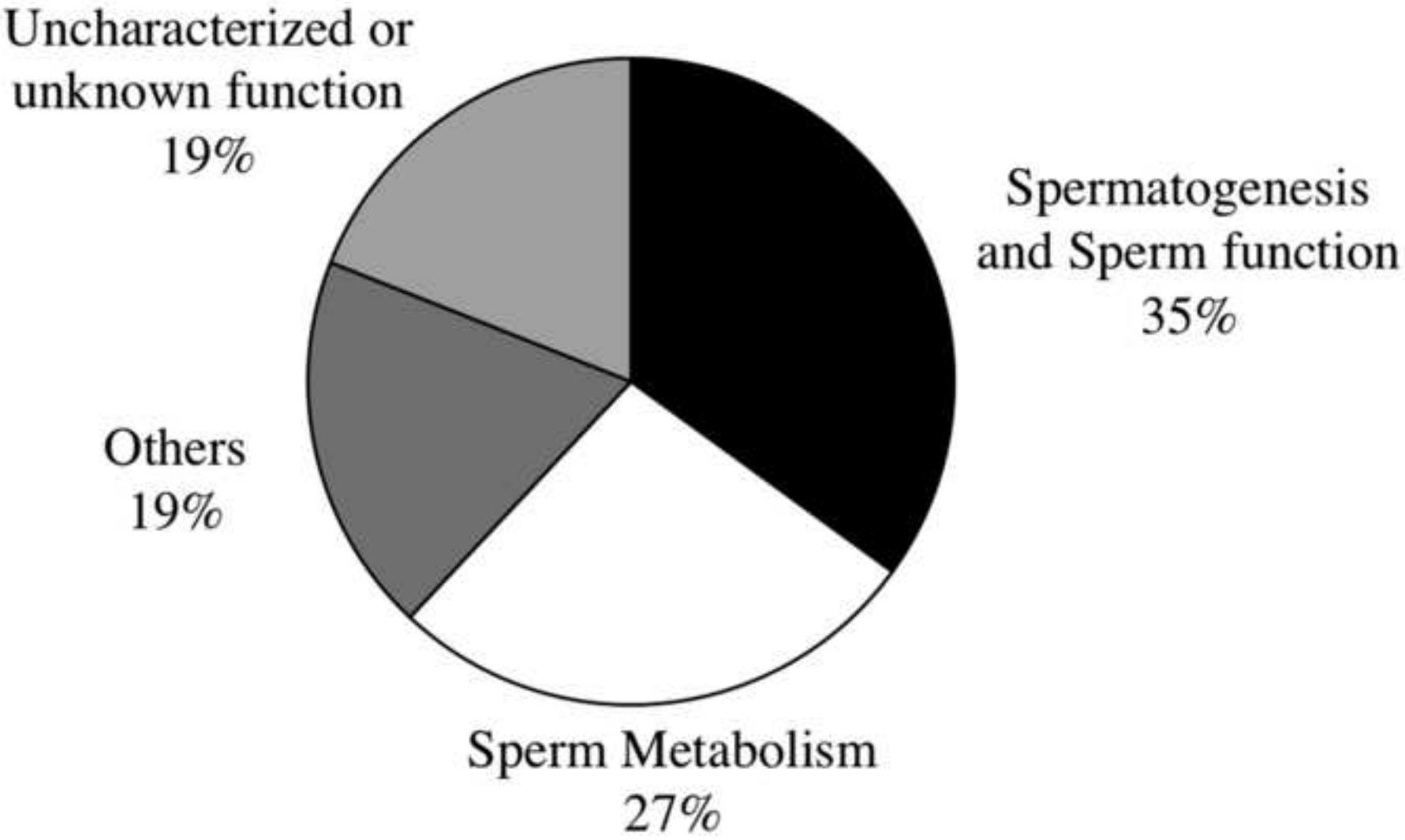

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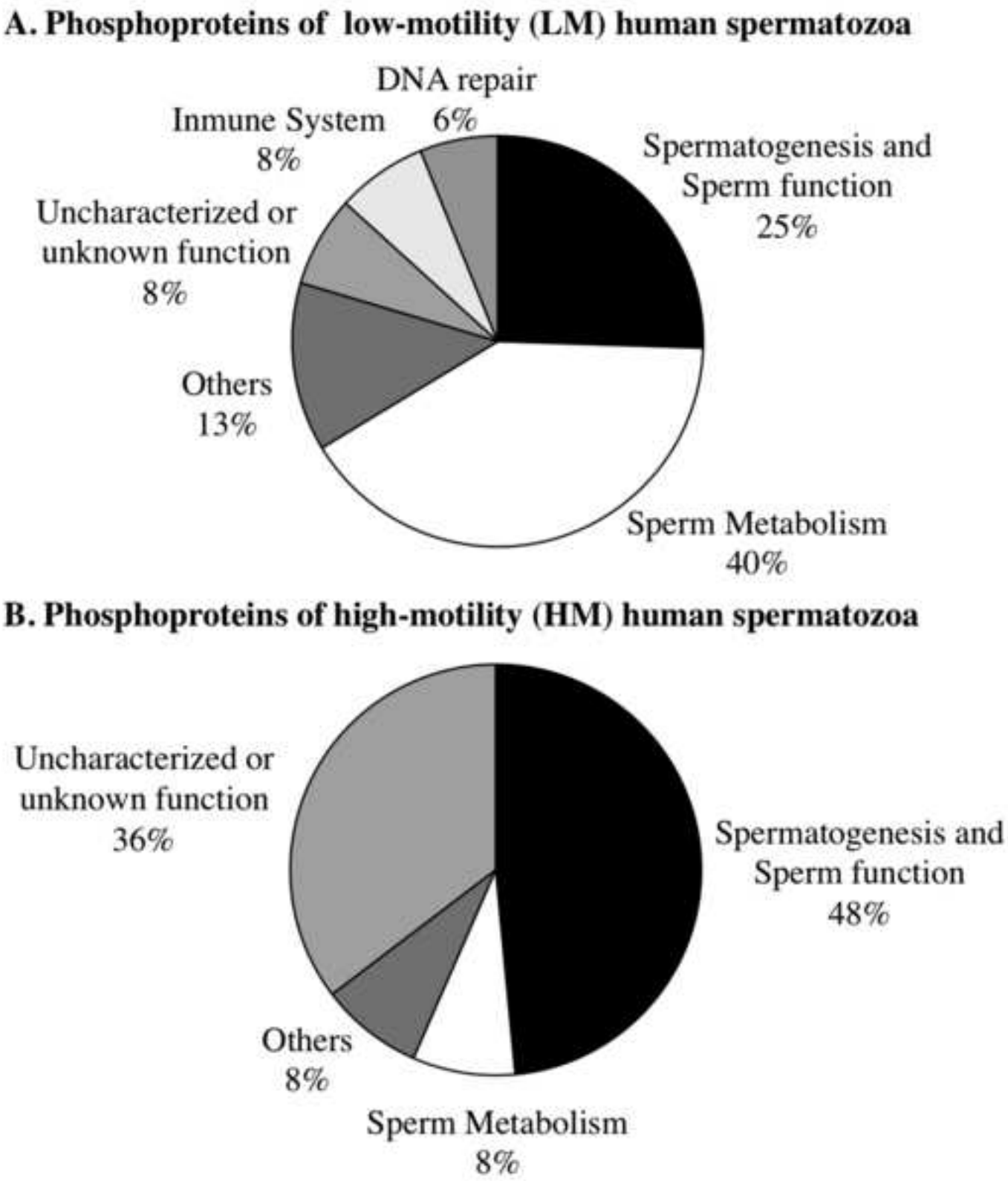

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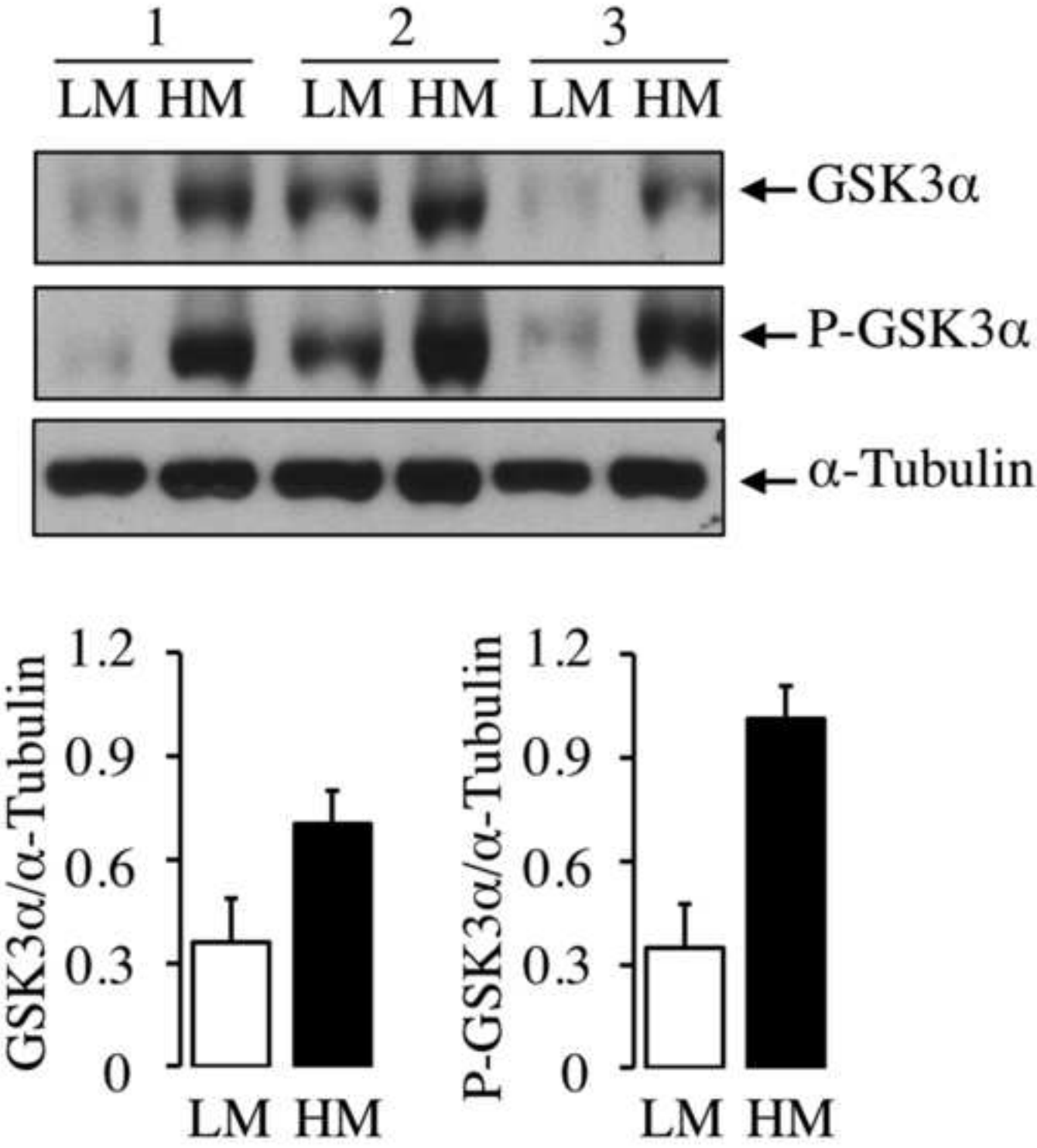

Supplementary material

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***Conflict of Interest**

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Author's contributions

D. Martin-Hidalgo and R. Serrano performed all experiments, contributed to data curation, formal analysis and wrote the manuscript.

C. Zaragoza contributed to funding acquisition.

L. J. Garcia- Marin and M. J. Bragado conceptualized and designed the study, analyzed and supervisedd the results, wrote the paper and contributed to funding acquisition.

All authors read and approved the final manuscript.