Original Paper

Cytoskeleton Structure in Mouse Sperm and Testes After 30 Days of Hindlimb Unloading and 12 Hours of Recovery

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Key Words

Weightlessness • Cytoskeleton • Sperm • Testes • Sperm-specific proteins

Abstract

Background/Aims: Changes in the external mechanical field result in cytoskeleton reorganization and the formation of adaptive patterns in different types of cells, including somatic cells and sex cells. The aim of this research was to study the protein and mRNA content of cytoskeletal and sperm-specific genes in the sperm and testis cells of mice. **Methods:** Mice were subjected to 30 days of antiorthostatic suspension to simulate weightlessness, followed by 12 h of recovery, while receiving essential phospholipids at a dosage of 500 mg/kg/day (30HSE and 30HSE+12h groups) or a similar dosage of a placebo (30HS and 30HS+12h groups). Accordingly, reference groups (CE group and C group) were formed. The total number and the percentage of motile spermatozoa were calculated using a Makler chamber. To analyze the number of viable spermatozoa and the permeability of their membranes, eosin staining was used as well as Diff-Quick for a morphological evaluation. Relative protein and mRNA content was estimated in a western blot and quantitative PCR assay, respectively. **Results:** The relative protein expression levels of actin (beta and gamma) and two alpha-actinin isoforms (1 and 4) remained constant in the sperm of all study groups, except for the 30HS+12h group, where the alpha-actinin-4 level was 13% higher than in the reference group ($p < 0.1$). In the testis cells, the relative actin isoform content was equivalent to that in the spermatozoa. However, in the testis cells, the *ACTN1* mRNA content was 17% higher in the 30HS group than in the C group ($p < 0.05$), and decreased after 12 h of recovery. In contrast, the *ACTN4* mRNA content was 20% lower in the 30HS group than in the reference group ($p < 0.05$) and increased after the 12-h recovery period. At the same time, in the group administered the essential phospholipids, the relative *ACTN1* and *ACTN4* mRNA content did not differ from those of the reference group. The relative beta-tubulin content was similar in the reference C group and the reference CE group, which was administered the essential phospholipids. In the 30HS and 30HS+12h groups, the beta-tubulin content decreased by 19% and 22% ($p < 0.05$),

respectively, and they also decreased in the groups administered the essential phospholipids (30HSE and 30HSE+12h groups, by 27% and 33%, respectively, $p < 0.05$). In the testis tissue, the relative tubulin content did not change in any of the experimental groups. At the same time, the relative mRNA content of the genes encoding the studied cytoskeletal proteins increased, which may indicate the protein content was regulated mainly at the translational level. **Conclusion:** The spermogram parameters and the content of the sperm-specific proteins and the associated mRNAs revealed a decrease in the number of mature spermatozoa in mice suspended under conditions of weightlessness. Moreover, the decrease was prevented by the administration of essential phospholipids.

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Introduction

The exploration of outer space is hampered not only by technical difficulties, but also by a number of medical problems, especially those associated with the negative effects of weightlessness, including issues at the cellular level. Nevertheless, the mechanisms underlying the interactions of a living cell with the physical field remain unclear.

In various types of cells that were cultured under the conditions of altered gravity, there are changes in the cellular profiles, disorganization of the microfilaments and sometimes the microtubules [1-6], and changes in the localization of the mitochondria [7], which is determined by the state of the intermediate filaments. In addition, changes have been reported in the expression levels of genes encoding cytoskeletal proteins and their associated signaling cascade participants [8-14]. Moreover, when the effects of microgravity are simulated in mesenchymal stem cells, osteogenic differentiation is inhibited, and adipogenic differentiation is activated [9-15], inhibiting the ability of embryonic stem cells to form embryoid bodies [16, 17]. Similar changes in the differentiation potential of stem cells under conditions of altered gravity may be associated with the reorganization of the cytoskeleton because its structures may participate in the selection of differentiation routes [18, 19]. Consequently, it can be assumed that the early stages of embryogenesis and gametogenesis, particularly in mammals, may be especially dependent on external mechanical conditions.

Pregnant rats spent 5 days aboard the Soviet satellites Kosmos-1129 (1979) and Kosmos-1514 (1983), and 11 days aboard the Space Shuttle during the second half of pregnancy in a joint experiment by Russian and American researchers (NIH-R1) (1994). Anatomical changes were not detected in any of these experiments, but the flight group animals exhibited accelerated development of the thyroid and parathyroid glands, decreases in mitotic activity and the number of cells in the thymus, and changes in the nervous system compared with control group animals on the ground [20-23]. It should be noted that rats are amniotic, and hence the development of embryos during the second half of pregnancy occurs in amniotic fluid, which can successfully dampen the changes in the external mechanical field. Additionally, the observed changes were associated with a change in the hormonal status of the females under the condition of weightlessness. When studying the early stages of development under the condition of weightlessness, mice were unable to become pregnant because pre-implanted embryos were aborted [24], possibly because of a change in the structure of the sex cells.

Space flight conditions decrease the number of mature spermatozoa in the testes of rats [25-27], indicating that the differentiation potential of precursor cells is changed, which decreases the efficiency of spermatogenesis [28]. Nevertheless, microgravity activates the cyclinB-cdc2 complex and the subsequent meiotic progression of isolated mouse spermatocytes from the mid-pachytene stage to the late-pachytene stage [29]. In sea urchin spermatozoa subjected to space flight conditions (STS-81, STS-84), phosphorylation of the FP130 protein, which is associated with the activation of mobility, occurs 3–4 times faster than under the condition of 1 g [30].

The published data indicate that changes in gravity have significant effects on the structure of cells and the pattern of gene expression, with similar changes observed for cells in culture and in tissue. The results of our previous studies revealed the formation of an adaptive pattern of cytoskeletal proteins in the muscle cells of rats and mice under the conditions of real space flight, modeled microgravity, and hypergravity [31-35]. Moreover, we have successfully used lecithin to prevent some changes in the structure of the cytoskeleton of muscle cells in rats undergoing antiorthostatic suspension [36].

At the same time, data on changes in male sex cells under microgravity conditions are very scarce. Therefore, we decided to evaluate the structure of the cytoskeleton of spermatozoa and, more generally, the testis cells of mice subjected to conditions simulating the effects of microgravity for a prolonged period. Mice were orally administered essential phospholipids to modify the membrane composition and, as a consequence, the structure of the cortical cytoskeleton.

Materials and Methods

Experimental design

The experiments were performed on the spermatozoa and testes of C57Black6/J mice ($n = 42$). To simulate microgravity, the standard Ilyin-Novikov model of antiorthostatic rodent suspension with the Moray-Holton modification was applied [37]. All animals received a standard rodent feed, and water was provided *ad libitum*. Half of the animals ($n = 21$) were randomly chosen to receive essential phospholipids in the form of Essentiale® Forte N (A. Nattermann and Cie. GmbH, Germany) at a dosage of 500 mg/kg/day for 10 days before the beginning of the experiment and for 30 days afterwards.

The following six groups were formed:

- C group ($n = 7$): reference group (mass = 28.2 ± 0.7 g);
- CE group ($n = 7$): reference group + essential phospholipids (mass = 28.4 ± 0.5 g);
- 30HS group ($n = 7$): suspension group (mass = 26.1 ± 0.9 g);
- 30HSE group ($n = 7$): suspension group + essential phospholipids (mass = 23.9 ± 0.9 g);
- 30HS+12h group ($n = 7$): group with a 12-h recovery period after the 30-day suspension period (mass = 26.0 ± 0.6 g);
- 30HSE+12h group ($n = 7$): group with a 12-h recovery period after the 30-day suspension period + essential phospholipids (mass = 25.2 ± 0.9 g).

After euthanizing the animals, the testes were extracted. The left testis was weighed and immediately frozen for subsequent protein and mRNA extractions. The caudal epididymis, from which the spermatozoa were extracted, was excised from the right testis.

All procedures with animals were approved by the Biomedical Ethics Committee of the State Scientific Center of the Russian Federation Institute of Biomedical Problems of the Russian Academy of Sciences (SSC RF-IMBP RAS; Protocol No. 425; June 20, 2016).

Sperm extraction and analysis of their motility and morphology

Immediately after extraction, the epididymis was placed in 1 ml α -MEM medium with 10% bovine serum heated to 37°C. Then, it was ground and incubated on a shaker (Thermo Shaker PST-60HL-4, Biosan, South Korea) for 30 min at 37°C. The resulting suspended material was filtered through nylon filters with a mesh diameter of 70 μ m. Some of the spermatozoa were frozen for subsequent protein and mRNA extractions. The motility and morphology of the remaining spermatozoa were analyzed.

To calculate the total number and the percentage of motile spermatozoa, a drop of the suspension was applied to a Makler chamber (Sefi Medical Instruments Ltd., Haifa, Israel) and observed in a phase-contrast microscope (Eclipse E200 MV, Nikon, Tokyo, Japan) with a magnification of 200 $\times$ (Fig. 1A-F). For each sample, the total number and the number of mobile spermatozoa in 100 squares of the Makler chamber were counted at least three times.

To analyze the number of viable spermatozoa and the permeability of their membranes, eosin staining was used. The stained cells were considered not viable, and the unstained cells were deemed viable (Fig. 1G–L). A minimum of 200 spermatozoa were studied for each sample under a light microscope with a magnification of 400×.

Spermatozoa smears were stained with Diff-Quick (Abris+, Moscow, Russia), which was used to determine the number of spermatozoa with normal morphology (Fig. 1M–R). For each sample, a minimum of 200 cells with normal and abnormal morphologies were examined by light microscopy with a magnification of 1,000×. On the basis of an earlier investigation by Gohari et al. [38], a morphological disorder was defined as any abnormality in sperm appearance or the head, tail, and remaining cytoplasmic residues. The average numbers of viable spermatozoa and spermatozoa with normal morphology were calculated as percentages.

Determination of protein content based on gel electrophoresis followed by immunoblotting

Proteins were extracted from frozen sperm and testis tissue. Cells and tissues were homogenized in Laemmli buffer containing a protease inhibitor cocktail (Calbiochem, San Diego, CA). Denaturing electrophoresis on polyacrylamide gels was performed using the Laemmli method (Bio-Rad Laboratories, Hercules, CA). Based on the measured concentration, an equal amount of protein was placed into each well, separated by electrophoresis, and transferred to a nitrocellulose membrane [39]. The efficiency of the protein transfer was assessed by Ponceau S staining before the membrane was blocked with milk. The following specific primary monoclonal antibodies were used at the dilutions recommended by the manufacturers to determine the expression levels of each protein: rabbit antibodies against JARID1B (KDM5B) diluted 1:100 (Bioss Antibodies, Woburn, MA), protamine diluted 1:250 (Sigma-Aldrich, Darmstadt, Germany), and SPACA3 diluted 1:1000 (OriGene, Rockville, MD); and mouse antibodies against beta-actin diluted 1:300, gamma-actin diluted 1:100, alpha-actinin-1 diluted 1:100, alpha-actinin-4 diluted 1:100, and beta-tubulin diluted 1:100 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Biotinylated goat antibodies were used as the secondary antibodies to detect rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and mouse IgG (Sigma-Aldrich) at dilutions of 1:10,000 and 1:20,000, respectively. Afterwards, all membranes were treated with a solution of streptavidin conjugated with horseradish peroxidase (Sigma-Aldrich) that was diluted 1:10,000. Protein bands were detected using 3,3'-diaminobenzidine (Merck, Burlington, MA). ImageJ software was used to analyze the obtained data.

Fig. 1. Typical pictures for sperm count, viability and morphology. (A – F) Counts under phase contrast (200×) for groups C, 30HS, 30HS+12h, CE, 30HSE, and 30HSE+12h, respectively. (G – L) Viability determined by eosin staining under light microscopy (400×) for groups C, 30HS, 30HS+12h, CE, 30HSE, and 30HSE+12h, respectively. (M – R) Viability determined by Diff-Quick staining under light microscopy with oil immersion (1000×) for groups C, 30HS, 30HS+12 h, CE, 30HSE, and 30HSE+12 h, respectively.

Table 1. Primer sequences and product sizes

Gene	Primer sequence, forward/reverse (5'...3')	Product size, bp
Actb (beta-actin)	gctgcgttttacacccttc/gtttgcctcaaccaactgct	218
Actg (gamma-actin)	ctggtgatctctgtgagca/tcaggagggaagaaccaga	184
Actn1 (alpha-actinin 1)	ggtcagcagcaacctcctc/tctttctccacctctctcca	167
Actn4 (alpha-actinin 4)	accctgaacagactcccttg/gatcgacaagcctcatctc	168
Tubb2b (beta-tubulin 2B)	ggcagcaagaagctaagag/cgaacacgaagtgtgtctggc	302
Kdm5b (lysine (K)-specific demethylase 5B, JARID1B)	agtggcttctctgttcgaga/aagcacatgccacatacaa	173
Prm1 (protamine 1)	atggccagataccgatgct/cgagatgctctgaagtctgg	231
Spaca3 (sperm acrosome-associated 3, SLLP1)	caaggccaaggtcttcagtc/tcagcttcgatgccacagc	150

Determination of mRNA content using quantitative PCR

Total RNA was isolated from frozen sperm and testis tissue using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for a subsequent targeted analysis of the mRNA content of the genes encoding a number of cytoskeletal proteins and spermatozoid-specific proteins. Reverse transcription was performed using d(T)₁₅ and 50 ng RNA for sperm and 500 ng RNA for testis tissue were used as templates. The abundance of the resulting cDNA was measured. To avoid artifacts, all samples were analyzed with at least three replicates. Real-time PCR was performed using primers designed using Primer3Plus software (Table 1) to assess the expression levels of the studied genes. The 2^{-ΔΔCt} method [40] was used to determine the expression level fold changes.

Statistical analysis

The protein and mRNA content data were statistically analyzed by analysis of variance and post hoc *t*-tests, and a significance level of *p* < 0.05 was applied to assess the reliability of differences between groups. Data are provided as the mean ± standard error.

Results

Testis mass

The weight of the left testis was not significantly different between reference groups C and CE, with weights of 259 ± 20 mg and 237 ± 23 mg, respectively. After the antiorthostatic suspension, as well as after the 12-h recovery period, the weight of the left testis was 27% lower in the 30HS group (188 ± 18 mg) and 32% lower in the 30HS+12h group (177 ± 16 mg) compared with the weight in the C group (both *p* < 0.05). A similar decrease was observed in the suspension and recovery groups administered essential phospholipids, with decreases of 34% (30HSE group, *m* = 171 ± 21 mg) and 36% (30HSE+12h group, *m* = 165 ± 15 mg) compared with the weight in the C group (both *p* < 0.05). There were no significant differences between the suspension groups with or without the 12-h recovery period.

Sperm motility and morphology

The number of sperm decreased significantly (*p* < 0.05) by 45% in the 30HS group and by 51% in the 30HS+12h group compared with the reference group data (Fig. 2). Moreover, the percentage of mobile sperm in these groups decreased more than 5-fold compared with that in the reference group.

The use of essential phospholipids resulted in a 34% increase in the number of mature spermatozoa in the CE group compared with the C group (*p* < 0.05). However, antiorthostatic suspension plus the administration of essential phospholipids with or without the 12-h recovery period did not result in a decrease in the number of mature spermatozoa. It should be noted that in the 30HSE group, the decrease in the percentage of mobile spermatozoa was similar to that in the 30HS group. Nevertheless, the spermogram parameters in the 30HSE+12h group did not differ from those in the reference group.

Motility parameters were normal and similar for all studied groups. The proportion of progressively motile spermatozoa was 31.2 ± 2.9% in the C group, 32.1 ± 2.8% in the 30HS group, 30.5 ± 2.6% in the 30HS+12h group, 33.0 ± 3.0% in the CE group, 32.3 ± 2.7% in the 30HSE group, and 31.4 ± 2.2% in the 30HSE+12h group.

Fig. 2. Sperm parameters. (A) Count. (B) Motility. (C) Viability. (D) Normal morphology. * $p < 0.05$ in comparison with the C group. ** $p < 0.05$ in comparison with the CE group.

Table 2. Distribution of the main spermatozoa morphological disorders

Part Group	Head (changes of shape, vesiculated head), %	Midpiece (distal and proximal vesicula), %	Tail (coiled tail), %
C	74 ± 8	8 ± 2	18 ± 4
30HS	78 ± 9	6 ± 2	16 ± 3
30HS+12h	75 ± 8	7 ± 3	18 ± 3
CE	79 ± 7	5 ± 2	16 ± 4
30HSE	72 ± 6	9 ± 3	19 ± 4
30HSE+12h	75 ± 7	6 ± 2	19 ± 3

In all studied groups, the percentages of viable spermatozoa and spermatozoa with normal morphology were correlated with the number of mobile spermatozoa. Most of the morphological changes were observed in the head (double-headed spermatozoa, elongated/circular head, misshapen head, and vesiculated acrosome), but we did not observe any changes in these distributions after unloading in comparison with the control group (Table 2).

Sperm-specific protein and mRNA content in the sperm and testis tissue

The relative content of JARID1B remained at the reference level in all groups, except for the 30HS group, in which it was 15% higher than in the reference group ($p < 0.05$) (Fig. 3A, B). The protamine (Fig. 3C, D) and SPACA3 (Fig. 3E, F) content remained unchanged in both spermatozoa and testis tissue after 30 days of antiorthostatic suspension and 12 h of recovery.

The relative *Jarid1B* mRNA content in the spermatozoa of the 30HS, 30HS+12h, and 30HSE groups was higher than the reference level by 114%, 101%, and 34%, respectively ($p < 0.05$) (Fig. 4A). At the same time, the *Jarid1B* mRNA content in the spermatozoa of the reference groups C and CE as well as the 30HSE+12h group did not differ. Similarly, in the

Fig. 3. Relative protein contents of sperm-specific proteins in the sperm (SP) and testis tissue (TE) with typical western blots. (A), (B) Jarid1B. (C), (D) Prm. (E), (F) Spaca3. SP, sperm; TE, testis tissue. * $p < 0.05$ in comparison with the C group. ** $p < 0.05$ in comparison with the CE group. # $p < 0.05$ in comparison with the 30HS group.

Fig. 4. Relative mRNA contents of genes encoding sperm-specific proteins in the sperm (SP) and testis tissue (TE). (A), (B) Jarid1B. (C), (D) Prm1. (E), (F) Spaca3. SP, sperm; TE, testis tissue. * $p < 0.05$ in comparison with the C group. ** $p < 0.05$ in comparison with the CE group. # $p < 0.05$ in comparison with the 30HS group.

testis tissue (Fig. 4B), the relative *Jarid1B* mRNA content in the 30HS, 30HS+12h, and 30HSE groups was 187%, 101%, and 54% higher than the reference level, respectively ($p < 0.05$), while the *Jarid1B* mRNA content in the C, CE, and 30HSE+12h groups did not differ.

Relative to the corresponding levels of the C group, the *Prm1* mRNA content in the spermatozoa (Fig. 4C) and the testis tissue (Fig. 4D) of the 30HS group was 80% and 59% lower, respectively (both $p < 0.05$), and they recovered to the reference levels after 12 h of recovery (30HS+12h group). In the groups administered essential phospholipids, the dynamics were the same, but the content in the reference CE group was lower than in the C group by 32% and 28% in the spermatozoa and testis tissue, respectively (both $p < 0.05$).

Similar to the *Prm1* mRNA content, the relative *Spaca3* mRNA content decreased by 46% in the spermatozoa (Fig. 4E) and by 31% in the testis tissue (Fig. 4F) after 30 days of suspension (30HS) (both $p < 0.05$), and after 12 h of recovery (30HS+12h), the levels

Fig. 5. Relative contents of microfilament proteins in the sperm (SP) revealed by typical western blots. (A) Beta-actin. (B) Gamma-actin. (C) Alpha-actinin-1. (D) Alpha-actinin-4. $^{\$}p < 0.1$ in comparison with the C group.

Fig. 6. Relative contents of microfilament proteins in the testis tissue (TE) revealed by typical western blots. (A) Beta-actin. (B) Gamma-actin. (C) Alpha-actinin-1. (D) Alpha-actinin-4. $^*p < 0.05$ in comparison with the C group. $^{\#}p < 0.05$ in comparison with the 30HS group.

recovered to the reference levels. The relative mRNA content was 37% lower in the spermatozoa and 23% lower in the testis tissue in the CE reference group compared with the C group (both $p < 0.05$). In the 30HSE suspension group, the *Spaca3* mRNA content increased to the reference level in the spermatozoa (Fig. 4E) and exceeded the reference level by 31% in the testis tissue ($p < 0.05$) (Fig. 4F). In the 30HSE+12h group, the *Spaca3* mRNA content in the spermatozoa decreased to the level of the CE group, while in the testis tissue, it decreased to the level of the C group.

Relative cytoskeletal protein content in sperm and testis tissue

The relative content of the actin isoforms ACTB (Fig. 5A), ACTG (Fig. 5B), and the actin-binding protein ACTN1 (Fig. 5C) remained the same in the spermatozoa of all study groups. The content of another actin-binding protein, ACTN4 (Fig. 5D), tended to increase by 13% in the 30HS+12h group compared with the C group ($p < 0.1$), while in groups administered essential phospholipids, it remained the same.

In the testis tissue, the relative content of the actin isoforms ACTB (Fig. 6A) and ACTG (Fig. 6B) was the same. The same was true in the spermatozoa. The ACTN1 (Fig. 6C) content was 17% higher in the 30HS group than in the C group ($p < 0.05$), and after 12 h of recovery, the level had decreased to the reference level. In contrast, the ACTN4 content (Fig. 6D) in the 30HS group was 20% lower than the reference level ($p < 0.05$), and increased to the reference level after 12 h of recovery. In groups administered essential phospholipids (30HSE and 30HSE+12h), the relative content of ACTN1 and ACTN4 did not differ from the reference levels.

The relative beta-tubulin content was similar in the reference C group and the reference group administered the essential phospholipids, CE. In the 30HS group after the 30-day suspension and in the 30HS+12h group after the 30-day suspension with a subsequent 12-h recovery period, the beta-tubulin content decreased by 19% and 22%, respectively ($p < 0.05$). In the similar groups administered essential phospholipids, 30HSE and 30HSE+12h, the beta-tubulin content decreased by 27% and 33%, respectively ($p < 0.05$) (Fig. 7A). In the testis tissue, the relative beta-tubulin content did not change in any of the experimental groups (Fig. 7B).

Relative mRNA content of genes that encode cytoskeletal proteins in sperm and testis tissue

The relative beta-actin mRNA content remained the same in the spermatozoa (Fig. 8A) and testis tissue (Fig. 8B) in all study groups.

The relative gamma-actin mRNA content did not differ between the spermatozoa of the 30HS group and those of the C group, while it was 54% higher in the spermatozoa of the 30HS+12h group than in the spermatozoa of the C group ($p < 0.05$). In the groups administered essential phospholipids (CE, 30HSE, and 30HSE+12h), the *Actg* mRNA content remained the same and did not differ from that of the C group (Fig. 8C). There was no change in the relative gamma-actin mRNA content in the testis tissue (Fig. 8D).

Fig. 7. Relative content of beta-tubulin revealed by typical western blots. (A) SP, sperm. (B) TE, testis tissue. * $p < 0.05$ in comparison with the C group. ** $p < 0.05$ in comparison with the CE group.

Fig. 8. Relative mRNA contents of genes encoding cytoskeletal proteins in the sperm (SP) and testis tissue (TE). (A), (B) Beta-actin. (C), (D) Gamma-actin. (E), (F) Alpha-actinin-1. (G), (H) Alpha-actinin-4. (I), (J) Beta-tubulin. SP, sperm; TE, testis tissue. * $p < 0.05$ in comparison with the C group. ** $p < 0.05$ in comparison with the CE group. # $p < 0.05$ in comparison with the 30HS group.

The *Actn1* mRNA content in the spermatozoa of the 30HS group was 47% higher than that of the C group ($p < 0.05$), and in the 30HS+12h group, it was 52% higher ($p < 0.05$). In the spermatozoa of the CE, 30HSE, and 30HSE+12h groups, the *Actn1* mRNA content was not significantly different, although it was 39%, 41%, and 31% higher, respectively, than in the spermatozoa of the C group (all $p < 0.05$) (Fig. 8E). In the testis tissue in the 30HS group, the *Actn1* mRNA content was 194% higher than in the reference group ($p < 0.05$), and after 12 h of recovery, it significantly decreased, although it still exceeded the reference level by 92% ($p < 0.05$) (Fig. 8F). As in the spermatozoa, the *Actn1* mRNA content was not significantly different in the testis tissue of the CE, 30HSE, and 30HSE+12h groups, but was 94%, 89%, and 91% higher, respectively, than in the testis tissue of the C group (all $p < 0.05$).

The relative *Actn4* mRNA content in the spermatozoa (Fig. 8G) and testis tissue (Fig. 8H) of the 30HS group did not differ from that of the C group. In the 30HS+12h group, it was 89% higher than the reference level in the spermatozoa and 88% higher than the reference level in the testis tissue (both $p < 0.05$). In the spermatozoa of the CE, 30HSE, and 30HSE+12h

groups, the *Actn4* mRNA content was the same as that in the spermatozoa of the C group (Fig. 8G). The *Actn4* mRNA content was 38%, 36%, and 42% higher in the testis tissue of the CE, 30HSE, and 30HSE+12h groups, respectively, than in the testis tissue of the C group (all $p < 0.05$) (Fig. 8H).

In the spermatozoa of the suspension and subsequent recovery groups, the relative beta-tubulin (*Tubb2b*) mRNA content was lower than the reference level (the levels in the C and CE groups did not differ) by 52% in the 30HS group, by 48% in the 30HS+12h group, by 49% in the 30HSE group, and by 46% in the 30HSE+12h group (all $p < 0.05$) (Fig. 8I). There was no change in the testis tissue, except in the 30HS+12h group, wherein the beta-tubulin mRNA content exceeded the reference level by 81% ($p < 0.05$) (Fig. 8J).

Discussion

Investigations into the effects of microgravity on the structural and functional states of sex cells and germinal tissue cells can help elucidate the effects of gravity on embryogenesis and the early development of animals. Therefore, in this study, we evaluated the cytoskeletal protein content and associated mRNA levels in the spermatozoa and testes of mice after a 30-day simulation of the effects of microgravity through antiorthostatic suspension, during which time, some of the mice were orally administered essential phospholipids. In addition, the resulting spermogram required the determination of the mRNA and protein content at different stages of spermatogenesis.

Testis weight and spermogram

After 30 days of antiorthostatic suspension, testis weight decreased and did not recover after 12 h. These changes could be caused by the combined effects of many factors, such as a lack of blood supply to the genital organs, primarily due to shifts in the fluid volume of the body in the cranial direction [41], as well as a change in hormonal regulation due to steroid hormones (testosterone) and gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]). However, the influence of the latter factor is unlikely because the 6-week suspension of male rats resulted in decreased testis weight and spermatogenesis; however, the levels of hormones controlling spermatogenesis (testosterone, FSH, and LH) remained within normal limits [27].

Nevertheless, our results also indicated that administering essential phospholipids may prevent a decrease in spermatogenesis. On the one hand, such a decrease may be due to an increase in apoptosis, which was observed after 7 days of antiorthostatic suspension [42]. On the other hand, a decrease in the number of mature spermatozoa may be associated with a change in the mRNA and protein levels of genes that control the differentiation of progenitor cells or the increase in apoptosis. Therefore, we estimated the relative protein and mRNA contents of the genes that were expressed at specific stages of spermatogenesis (genes encoding JARID1B for spermatogonia, protamine for spermatids, and SPACA3 for mature spermatozoa).

Sperm-specific proteins and their expression levels

The histone demethylase that demethylates “Lys-4” of histone H3, namely PLU-1/KDM5B/JARID1B, is expressed in various cell types such as hematopoietic stem cells [43], neural precursors, and embryonic stem cells [44]. Moreover, it is highly expressed in the mitotic spermatogonia [45, 46]. That is why we estimated its content as a marker of spermatogonia stem cells. Genes with expression levels stimulated by the JARID1B protein are involved in the maintenance of proliferation processes and the suppression of the expression of the genes responsible for terminating the cell cycle. It is believed that the differentiation of spermatogonia into spermatocytes is associated with the inactivation of the *Jarid1B* gene [47]. Our findings indicate that the JARID1B protein and associated mRNA content is higher after antiorthostatic suspension than in the reference group. This suggests there is a

decrease in the cell differentiation potential and a cell cycle arrest in spermatogonia, which in turn, can lead to the decrease in the number of mature spermatozoa that we observed in the 30HS group. In this case, after 12 h of recovery, their expression levels returned to the reference level.

We used protamines as spermatid markers [48]. Protein content remained unchanged in all study groups, but the relative mRNA content decreased in the suspension groups and was restored to the reference level after a 12-h recovery period. We can assume that such regulation is carried out at the translational level, apparently contributing to the normalization of the protein pattern because of its exceptional physiological effects. Protamines are necessary for the high packing density of chromosomes, which minimizes the size and improves the hydrodynamic properties of the mature spermatozoon [48]. A decrease in the protamine content can be extremely important in early embryogenesis, leading to the formation of an abnormal pattern of gene expression, because it is assumed that after the fusion of the gametes, only genes with promoters and enhancers linked to histone proteins are initially expressed [49, 50]. Thus, these results suggest that the chromatin structure may be changed in response to antiorthostatic suspension. It is well known that under conditions of real space flight and various microgravity models, oxidative stress occurs due to the accumulation of reactive oxygen species and nitrogen oxide [51]. In turn, oxidative stress is one of the most well-studied factors leading to chromatin damage in spermatozoa [52, 53], especially if the hormone concentrations are stable, there is a lack of toxic effects, and so on. Nevertheless, we did not observe a redistribution of morphological abnormalities after suspension, and the proportion of spermatozoa with an abnormal head did not differ in all study groups. However, we considered all spermatozoa with an abnormal head, without evaluating the proportion of double headed spermatozoa, misshapen heads, etc. Thus, an increase in the number of spermatozoa with damaged chromatin was not excluded. However, this hypothesis can only be confirmed by an explicit evaluation of the integrity of the chromatin (for example, by using the TUNEL method and HALO-SPERM method).

As well as protamines, sperm lysozyme-like protein 1 (SLLP1, SPACA3) contributes greatly to fertility. It participates in the fusion of gametes [54] and could be a marker of mature spermatozoa. The relative abundance of the SPACA3 protein, which is localized in the acrosomal vesicle of mature spermatozoa, was unchanged in all study groups. At the same time, its mRNA content decreased in the 30 HS group but was restored to the reference level after a 12-h recovery. This supports the hypothesis that the regulation may occur at the translational level.

Thus, the spermogram was correlated with the changes in the protein and mRNA content of the corresponding genes, the expression levels of which were typical for various stages of spermatogenesis; however, the reasons for such changes and, especially, the reasons for the recovery of the expression to reference levels after 12 h of recovery remain unclear. It can be assumed that a change in the orientation of the genital organs in the field of gravity and fluid shift, as well as the consequent change in the external mechanical stress, can result in an intracellular response and, therefore, a change in the gene expression pattern.

Previously, we assumed that the initial acts of mechanotransduction may be associated with the dissociation of the actin-binding proteins from the cortical cytoskeleton, such that with a decrease in external mechanical stress, alpha-actinin-4 may migrate, and in the case of an increase, alpha-actinin-1 may migrate. On the basis of the above evidence, we decided to evaluate the content of various cytoskeletal proteins and the associated mRNA.

mRNA and protein expression levels of cytoskeletal genes

The total beta- and gamma-actin protein content, as well as the mRNA content of the corresponding genes, did not change significantly relative to the levels in the post-suspension and recovery reference groups. However, the abundance of the actin-binding proteins and, especially, the mRNA content of the corresponding genes did change. In the testes, the alpha-actinin-1 protein content after suspension was higher than in the reference group and decreased after a 12-h recovery period, as did the content of the corresponding

mRNA. Conversely, the alpha-actinin-4 protein content decreased after suspension and then increased to the reference level after a 12-h recovery period. In the spermatozoa, an increase in the alpha-actinin-4 protein content was observed only after a 12-h recovery period; however, changes in the mRNA content of actin-binding proteins were similar to those observed in the testes. Alpha-actinin-4 interacts with the focal-adhesive complex, ensuring it is connected to the actin cytoskeleton [55]. This connection supports the activity of the focal-adhesive kinase, which provides the acrosome the integrity necessary for capacitation [56]. Consequently, changes in the alpha-actinin-4 content in spermatozoa can have significant consequences for their fertilization ability.

At the same time, there was no change in the tubulin cytoskeleton in the testes as well as in other types of cells during changes in gravity [36]. In contrast, in the spermatozoa, there were decreases in the protein and mRNA content that did not recover after a 12-h recovery period. A decrease in the tubulin content can result in problems with the differentiation of male germ cells because tubulin participates in the formation of the cleavage spindle during cell division [57], which, along with changes in the expression patterns of the sperm-specific genes, can result in a shift of the cellular profile towards immature forms. In addition, a decrease in the number of tubulin molecules can cause the formation of defective microtubules in the tail of the spermatozoon and lead to defective motility, as well as the disruption of the acrosomal reaction during fertilization [58]. However, we did not observe any changes to the motility characteristics (about 30–35% of spermatozoa exhibited progressive motility) or the redistribution of tail morphology abnormalities. We suggest that decreasing tubulin content was compensated by an increase in the efficiency of microtubule assembly in the axonema of the sperm tail, possibly at the expense of other cellular structures. This is only a suggestion and needs to be supported by direct evidence of changes in spermatozoon structure, especially the flagellum (maybe by electron microscopy).

It should be noted that the changes in the actin-binding protein content were more pronounced in the testis tissue than in the spermatozoa. Minor changes in the spermatozoa, with the exception of changes in tubulin, can be associated with a fundamentally different cell structure that is significantly more compact and, as a consequence, less susceptible to changes in external stress from a mechanical point of view. At the same time, an analysis of testis tissue revealed different types of cells. Most of these cells have developed cortical cytoskeletons and normal cytoplasm volumes, which may result in the dissociation of the actin-binding proteins and the initiation of intracellular signaling pathways to form the observed adaptive pattern of gene expression.

Effect of the administration of essential phospholipids

Administration of the essential phospholipids can lead to a decrease in the cholesterol content of different cell types [59]. It is well known that the cholesterol content in the lipid microdomains of plasma membranes (rafts) can modify the activity of ionic channels and the organization of the actin network [60–68]. Furthermore, a decrease in the cholesterol raft content in the membrane can result in a change in the ratio between the proteins anchored in the membrane and the proteins in the cytoplasm [69–70], with a significant effect on the expression of the genes encoding them. Thus, it can explain a phenomenon we observed in muscle cells [36] as well as in this study. Specifically, the relative content of mRNAs corresponding to the actin-binding proteins was higher in the reference group administered the essential phospholipids than in the reference group not administered the essential phospholipids.

Our previous measurements [36] showed that even unsaturated phosphatidylcholines (such as lecithin) can decrease the cytochrome *c* content in the cytoplasmic fraction. Such a decrease may lead to a decrease in apoptosis [72]. The administration of essential phospholipids prevented changes in the JARID1B protein content and decreases in the spermatozoa count after hindlimb unloading, maybe due to a decreasing apoptosis level. However, the use of phospholipids did not prevent the decrease in motility, viability, and proportion of spermatozoa with normal morphology after suspension, but these parameters

were restored after 12 h of recovery. Because most morphological changes are associated with spermatozoon head abnormalities (especially vesiculation of the acrosome), we can assume that a rapid recovery within 12 h may be associated with the altered phospholipid composition of the sperm membrane. Additionally, *Spaca3* expression increased in the 30HSE group instead of decreasing like in the 30HS group. It should be noted that the acrosomal reaction in mammals, which is mediated by SPACA3, is impossible without capacitation, one of the stages in which the cholesterol content in the sperm membrane decreases [73, 74].

Despite the increasing spermatozoa count following the administration of essential phospholipids and a very short recovery period after gravitational unloading, we cannot say anything about their fertilization ability. However, it should be mentioned that strong evidence might be obtained from an experiment that estimates the acrosome reaction (for example, by acrosome reaction ionophore challenge) and *zona pellucida* binding.

Conclusion

The results obtained in this study indicate that following a 30-day antiorthostatic suspension and 12-h recovery, there were practically no changes in the expression patterns of cytoskeletal proteins and sperm-specific proteins in the spermatozoa of mice, except for a decrease in the tubulin protein content and an increase in the JARID1B protein content after the suspension. However, the relative mRNA content of the genes encoding cytoskeletal proteins and sperm-specific proteins did change. These data suggest that the regulation of protein expression patterns in spermatozoa under the conditions of a modified external mechanical field is mainly carried out at the translational level. In general, we observed multidirectional changes in the actin-binding protein content (alpha-actinin-1 and alpha-actinin-4) in the testes that were similar to those for other types of cells. The oral administration of phospholipids helped to prevent these changes and restore the spermogram parameters to the normal range within 12 h; however, in the reference group, the administration of phospholipids resulted in an increase in the expression of cytoskeletal genes, a phenomenon that requires additional investigation. In addition, the oral administration of phospholipids leads to an increase in the number of spermatozoa in control mice, which also requires further study.

Limitations of this study were related to the phospholipid effects on spermogram parameters and sperm-specific gene expression. The rapid recovery of spermogram parameters within 12 h after a 30-day antiorthostatic suspension with phospholipids being administered should be characterized further in future studies focused on the chromatin state and fertilization ability of spermatozoa in general.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

References

- 1 Sakar D, Nagaya T, Koga K, Seo H: Culture in Vector-averaged Gravity Environment in a Clinostat Results in Detachment of Osteoblastic ROS 17/2.8 Cells. *Environmental Medicine* 1999;43:22–24.
- 2 Uva BM, Masini MA, Sturla M, Prato P, Passalacqua M, Giuliani M, Tagliaferro G, Strollo F: Clinorotation-induced weightlessness influences the cytoskeleton of glial cells in culture. *Brain Res* 2002;934:132–139.
- 3 Gaboyard S, Blachard MP, Travo BT, Viso M, Sans A, Lehouelleur J: Weightlessness affects cytoskeleton of rat utricular hair cells during maturation *in vitro*. *NeuroReport* 2002;13:2139–2142.
- 4 Plett PA, Abonour R, Frankovitz SM, Orschell CM: Impact of modeled microgravity on migration, differentiation and cell cycle control of primitive human hematopoietic progenitor cells. *Experimental Hematology* 2004;32:773–781.
- 5 Kacena MA, Todd P, Landis WJ. Osteoblasts subjected to spaceflight and simulated space shuttle launch conditions. *In vitro Cell Dev Biol – Animal* 2004;39:454–459.
- 6 Crawford-Young SJ: Effects of microgravity on cell cytoskeleton and embryogenesis. *Int J Dev Biol* 2006;50:183–191.
- 7 Schatten H, Lewis ML, Chakrabarti A: Spaceflight and clinorotation cause cytoskeleton and mitochondria changes and increases in apoptosis in cultured cells. *Acta Astronautica* 2001;49:399–418.
- 8 Gershovich PM, Gershovich JG, Buravkova LB: Simulated microgravity alters actin cytoskeleton and integrin-mediated focal adhesions of cultured human mesenchymal stromal cells. *J Grav Physiol* 2008;15:203–204.
- 9 Zayzafoon M, Gathings WE, McDonald JM: Modeled microgravity inhibits osteogenic differentiation of human mesenchymal stem cells and increases adipogenesis. *Endocrinology* 2004;145:2421–2432.
- 10 Meyers VE, Zayzafoon M, Gonda SR, Gathings WE, McDonald JM: Modeled microgravity disrupts collagen I/ integrin signaling during osteoblastic differentiation of human mesenchymal stem cells. *Journal of Cellular Biochemistry* 2004;93:697–707.
- 11 Meyers VE, Zayzafoon M, Douglas JT, McDonald JM: RhoA and cytoskeletal disruption mediate reduced osteoblastogenesis and enhanced adipogenesis of human mesenchymal stem cells in modeled microgravity. *Journal of Bone and Mineral Research* 2005;20:1858–1866.
- 12 Dai ZQ, Wang R, Ling SK, Wan YM, Li YH: Simulated microgravity inhibits the proliferation and osteogenesis of rat bone marrow mesenchymal stem cells. *Cell Proliferation* 2007;40:671–684.
- 13 Patel MJ, Liu W, Sykes MC, Ward NE, Risin SA, Risin D, Jo H: Identification of mechanosensitive genes in osteoblasts by comparative microarray studies using the rotating wall vessel and the Random Positioning Machine. *Journal of Cellular Biochemistry* 2007;101:587–599.
- 14 Pan Z, Yang J, Guo C, Shi D, Shen D, Zheng Q, Chen R, Xu Y, Xi Y, Wang J: Effects of hindlimb unloading on *ex vivo* growth and osteogenic/adipogenic potentials of bone marrow derived mesenchymal stem cells in rats. *Stem Cells and Development* 2008;17:795–804.
- 15 Huang Y, Dai ZQ, Ling SK, Zhang HY, Wan YM, Li YH: Gravity, a regulation factor in the differentiation of rat bone marrow mesenchymal stem cells. *Journal of Biomedical Science* 2009;16:87, 14 pages.
- 16 Buravkova LB, Romanov YA, Konstantinova NA, Buravkov SV, Gershovich YG, Grivennikov IA: Cultured stem cells are sensitive to gravity changes. *Acta Astronautica* 2008;63:603–608.
- 17 Konstantinova NA, Buravkova LB, Manuilova ES, Grivennikov IA: The effects of gravity vector randomization on mouse embryonic stem cells *in vitro*. *Journal of Gravitational Physiology* 2006;13:149–150.
- 18 Sordella R, Jiang W, Chen GC, Curto M, Settleman J: Modulation of Rho GTPase signaling regulates a switch between adipogenesis and myogenesis. *Cell* 2003;113:147–158.
- 19 McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS: Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Developmental Cell* 2004;6:483–495.
- 20 Ontogeny of Mammals in Microgravity, NASA TM 103978, 1993.
- 21 Serova LV, Natochkin IV, Nosovskii AM, Shakhmatova EI, Fast T: Effect of weightlessness on the mother-fetus system (results of embryological experiment NIH-R1 abroad the “Space Shuttle”. *Aviakosm. Ekol. Med* 1996;30:4–8.
- 22 Savel’ev SV, Serova LV, Besova NV, Nosovskii AM: Effect of weightlessness on rats endocrine system development. *Aviakosm. Ekol. Med* 1998;32:31–36.

- 23 Serova LV. Microgravity and development of the mammals: problems, results, prospects. *Aviakosm. Ekol. Med* 2001;35:32–35.
- 24 Kojima Y, Sasaki S, Kubota Y, Ikeuchi T, Hayashi Y, Kohri K: Effects of simulated microgravity on mammalian fertilization and preimplantation embryonic development *in vitro*. *Fertil. Steril* 2000;74:1142–1147.
- 25 Serova LV. Effect of weightlessness on the reproductive system of mammals. *Kosm. Biol. Aviakosm. Med* 1989;23:11–16.
- 26 Serova LV, Denisova LA, Apanasenko ZI, Kuznetsova MA, Meizerov ES. Reproductive function of the male rat after a flight on the Kosmos-1129 biosatellite. *Kosm. Biol. Aviakosm. Med* 1982;16:62–65.
- 27 Tash JS, Johnson DC, Enders GC: Long-term (6wk) hindlimb suspension inhibits spermatogenesis in adult male rats. *J. Appl. Physiol* 2002;92:1191–1198.
- 28 Pellegrini M, Di Siena S, Claps G, Di Cesare S, Dolci S, Rossi P, Geremia R, Grimaldi P: Microgravity promotes differentiation and meiotic entry of postnatal mouse male germ cells. *PLoS ONE* 2010;5:e9064.
- 29 Di Agostino S., Botti F., Di Carlo A., Sette C., Geremia R: Meiotic progression of isolated mouse spermatocytes under simulated microgravity. *Reproduction* 2004;128:25–32.
- 30 Tash JS, Bracho GE: Microgravity alters protein phosphorylation changes during initiation of sea urchin sperm motility. *FASEB J* 1999;13:S43–54.
- 31 Ogneva IV: The transversal stiffness of fibers and the desmin content in the leg muscles of rats under gravitational unloading of various duration. *J Appl Physiol* 2010;109:1702–1709.
- 32 Ogneva IV: Cell mechanosensitivity: mechanical properties and interaction with gravitational field. *BioMed Research International* 2013;Article ID:598461, 17 pages.
- 33 Ogneva IV, Biryukov NS, Leinsoo TA, Larina IM: Possible role of non-muscle alpha-actinins in muscle cell mechanosensitivity. *PLoS ONE* 2014;9:e96395.
- 34 Ogneva IV, Maximova MV, Larina IM: Structure of cortical cytoskeleton in fibers of mouse muscle cells after being exposed to a 30-day space flight on board the BION-M1 biosatellite. *J Appl Physiol* 2014;116:1315–1323.
- 35 Ogneva IV, Gnyubkin V, Laroche N, Maximova MV, Larina IM, Vico L: Structure of the cortical cytoskeleton in fibers of postural muscles and cardiomyocytes of mice after 30-day 2g-centrifugation. *J Appl Physiol* 2015;118:613–623.
- 36 Ogneva IV, Biryukov NS: Lecithin prevents cortical cytoskeleton reorganization in rat soleus muscle fibers under short-term gravitational disuse. *PLoS ONE* 2016;11:e0153650.
- 37 Morey-Holton E, Globus RK, Kaplansky A, Durnova G: The hindlimb unloading rat model: literature overview, technique update and comparison with spaceflight data. *Adv Space Biol Med* 2005;10:7–40.
- 38 Gohari FA, Saranjam B, Asgari M, Omidi L, Ekrami H, Moussavi-Najarkola SA: An experimental study of the effects of combined exposure to microwave and heat on gene expression and sperm parameters in mice. *J Hum Reprod Sci* 2017;10:128–134.
- 39 Towbin H, Staehlin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc Natl AcadSci USA* 1970;76:4350–4354.
- 40 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 2001;2:402–408.
- 41 Watenpaugh DE, Hargens AR: The cardiovascular system in microgravity. In *Handbook of Physiology. Environmental Physiology*, Am Physiol Soc, 4(vol. I, pt. 3, chapt. 29) 1996;631–674.
- 42 Kamiya H, Sasaki Sh, Ikeuchi T, Umemoto Y, Tatsura H, Hayashi Y, Kaneko Sh, Kohri K: Effect of simulated microgravity on testosterone and sperm motility in mice. *J Androl* 2003;24:885–890.
- 43 Stewart MH, Albert M, Sroczynska P, Cruickshank VA, Guo Y, Rossi DJ, Helin K, Enver T: The histone demethylase Jarid1b is required for hematopoietic stem cell self-renewal in mice. *Blood* 2015;125:2075–2078.
- 44 Schmitz SU, Albert M, Malatesta M, Morey L, Johansen JV, Bak M, Tommerup N, Abarrategui I, Helin K: Jarid1b targets genes regulating development and it is involved in neural differentiation. *EMBO J* 2011;30:4586–4600.
- 45 Madsen B, Tarsounas M, Burchell JM, Hall D, Poulson R, Taylor-Papadimitriou J: PLU-1, a transcriptional repressor and putative testis-cancer antigen, has a specific expression and localization pattern during meiosis. *Chromosoma* 2003;112:124–132.

- 46 Cui Y, Zhang Y, Wei Zh, Gao J, Yu T, Chen R, Lv X, Pan Ch: Pig KDM5B: mRNA expression profiles of different tissues and testicular cells and association analyses with testicular morphology traits. *Gene* 2018;650:27–33.
- 47 Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. *Nat. Rev. Cancer* 2005;5:615–625.
- 48 Depa-Martynow M, Kempisty B, Lianeri M, Jagodzinski PP, Jedrzejczak P: Association between fertilin beta, protamines 1 and 2 and spermatid-specific linker histone H1-like protein mRNA levels, fertilization ability of human spermatozoa, and quality of preimplantation embryos. *Folia Histochem Cytobiol.* 2007;45:S79–85.
- 49 Aoki VW, Liu L, Carrell DT: A novel mechanism of protamine expression deregulation highlighted by abnormal protamine transcript retention in infertile human males with sperm protamine deficiency. *Mol. Hum. Reprod* 2006;12:41–50.
- 50 Balhorn R: The protamine family of sperm nuclear proteins. *Genome Biol* 2007;8:227, 8 pages.
- 51 Goodwin ThJ, Christofidou-Solomidou M: Oxidative stress and space biology: an organ-based approach. *Int J Mol Sci* 2018;19:959–969.
- 52 Omu AE: Sperm parameters: paradigmatic index of good health and longevity. *Med Princ Prac* 2013;22:30–42.
- 53 Gatimel N, Moreau J, Parinaud J, Leandri RD: Sperm morphology: assessment pathophysiology, clinical relevance and state of the ART in 2017. *Andrology* 2017;5:845–862.
- 54 Lea IA, Widgren EE, O’Rand MG: Association of sperm protein 17 with A-kinase anchoring protein 3 in flagella. *Reprod Biol Endocrinol* 2004;2:57, 7 pages.
- 55 Baron MD, Davison MD, Jones P, Critchley DR: The structure and function of alpha-actinin. *Biochem Soc Trans* 1987;15:796–798.
- 56 Roa-Espitia AL, Hernandez-Rendon ER, Baltierrez-Hoyos R, Munoz-Gotera RJ, Cote-Velez A, Jimenez I, Gonzalez-Marquez H, Hernandez-Gonzalez EO: Focal adhesion kinase is required for actin polymerization and remodeling of the cytoskeleton during sperm capacitation. *Biol. Open* 2016;5:1189–1199.
- 57 Fackenthal JD, Turner FR, Raff EC: Tissue-specific microtubules functions in *Drosophila* spermatogenesis require the beta 2-tubulin isotype-specific carboxy terminus. *Dev Biol* 1993;158:213–227.
- 58 Xia J, Reigada D, Mitchell CH, Ren D: CATSPER channel-mediated Ca²⁺ entry into mouse sperm triggers a tail-to-head propagation. *Biol. Reprod* 2007;77:551–559.
- 59 Jayaraman T, Kannappan S, Ravichandran MK, Anuradha CV: Impact of Essentiale L on ethanol induced changes in rat brain and erythrocytes. *Singapore Med J* 2008;49:320–327.
- 60 Harder T, Simons K: Clusters of glycolipid and glycosylphosphatidylinositol-anchored proteins in lymphoid cells: accumulation of actin regulated by local tyrosine phosphorylation. *Eur J Immunol* 1999;29:556–562.
- 61 Brown DA, London E: Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J Biol Chem* 2000;275:17221–17224.
- 62 Nebel T, Pestonjamas KN, Leszyk JD, Crowley JL, Oh SW, Luna EJ: Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes. *J Biol Chem* 2002;277:43399–43409.
- 63 Brown DA: Lipid rafts, detergent-resistant membranes, and raft targeting signals. *Physiology (Bethesda)* 2006;21:430–439.
- 64 Levitan I, Christian AE, Tulenko TN, Rothblat GH: Membrane cholesterol content modulates activation of volume-regulated anion current in bovine endothelial cells. *J Gen Physiol* 2000;115:405–416.
- 65 Shlyonsky VG, Mies F, Sariban-Sohraby S: Epithelial sodium channel activity in detergent-resistant membrane microdomains. *Am J Physiol Renal Physiol* 2003;284:F182–188.
- 66 Edidin M: The state of lipid rafts: from model membranes to cells. *Annu Rev Biophys Biomol Struct* 2003;32:257–283.
- 67 Chubinskiy-Nadezhdin VI, Negulyaev YA, Morachevskaya EA: Cholesterol depletion-induced inhibition of stretch-activated channels is mediated via actin rearrangement. *Biochem Biophys Res Commun* 2011;412:80–85.
- 68 Morachevskaya EA, Sudarikova AV, Negulyaev YA: Mechanosensitive channel activity and F-actin organization in cholesterol-depleted human leukaemia cells. *Cell Biol Int* 2007;31:374–381.
- 69 Harder T, Simons K: Clusters of glycolipid and glycosylphosphatidylinositol-anchored proteins in lymphoid cells: accumulation of actin regulated by local tyrosine phosphorylation. *Eur J Immunol* 1999;29:556–562.

- 70 Brown DA, London E: Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J Biol Chem* 2000;275:17221–17224.
- 71 Brown DA: Lipid rafts, detergent-resistant membranes, and raft targeting signals. *Physiology (Bethesda)* 2006;21:430–439.
- 72 Mak KM, Wen K, Ren Ch, Lieber ChS: Dilinoleoylphosphatidylcholine reproduces the antiapoptotic actions of polyenylphosphatidylcholine against ethanol-induced hepatocyte apoptosis. *Alcohol Clin Exp Res* 2003;27:997–1005.
- 73 Davis BK: Inhibition of fertilizing capacity in mammalian spermatozoa by natural and synthetic vesicles; in J.J. Kabara (eds): *Symposium of the Pharmacological Effect of Lipids*. Amer. Oil Chem Soc, Champaign, IL, 1978, pp 145–157.
- 74 Cross NL: Role of cholesterol in sperm capacitation. *Biol. Reprod* 1998;59:7–11.