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Doctoral dissertation resume

INCREASING CRYOPRESERVING CAPACITY OF EJACULATE MEDIA FOR OVCHEPOLSKA PRAMENKA BY UTILIZING SEMINAL PLASMA AND GLUTATHIONE

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ABSTRACT

Specific protein fractions of ram seminal plasma (HSP) have ability to adhere to structurally damaged regions of cell membrane. Reduced glutathione (GSH) is one of the main antioxidative components of HSP which regulates the peroxidative processes, preventing oxidative stress of spermatozoa. These two components have been used as additives in ejaculate media (EM), in aim of increasing cryopreserving (CP) success of ram spermatozoa, but scientific research reports have conflicting findings. In aim of confirming and/or rejecting these claims, the following aims have been set: 1.) To assess the CP success of EM, supplemented with GSH and/or HSP, used prior (a-CT) or following CP (b-CT); 2.) To assess the effect of post-thawing incubation time on achieving higher CP success of supplemented EM. We used fresh ejaculates (ovchepolska pramenka) which were pooled following qualitative and quantitative assessment. Eight aliquots (Alq1-8) were suspended with control (C) and experimental (E) EM: Alq1-5 with C (HSP/GSH free), Alq6 with E1 (GSH-5mM), Alq7 with E2 (HSP-20%), and Alq8 with E3 (GSH-5mM+HSP-20%). Following CP in liquid nitrogen, Alq1-4 samples were resuspended (x2) in consecutive order with C, E1, E2 and E3 until reaching final cell concentration as the remaining Alq samples ($50x10^6$/ml). Each Alq samples (n=25) were incubated at 37°C following CP and were analyzed in groups which were assigned according to used EM, suspension method and incubation time (0- and 3-hours). CP success was assessed according to the structural integrity, morphology (Hancock-2), and motility parameters (CASA) of thawed spermatozoa. Values were presented in distribution frequency (%) of parameter categories, organized in contingency tables, analyzed with χ^2 of independence. The significance of the test and respective values was analyzed according to α-critical value, degrees of freedom and relative contribution indexes (RCI) which were correlated with higher/lower than expected frequency (f+/-). E2-a-0 and E3-a-0 samples had >RCI for f+ category with intact cell integrity and morphology, while E2-b-0 and E3-b-0 did not have significantly different RCIs than C-b-0. E3-a-3 had >RCI for f+ in motility parameter categories which characterized the motility as un-capacitated. E1-a and E1-b groups had <RCI for these categories or had no effect on f+. Following these results, it can be summarized that the addition of control or experimental EM following thawing has higher effect on achieving larger number of static or capacitated motility spermatozoa with affected structural integrity. The pre-CP addition of EM supplemented with HSP and HSP/GSH increased the observed number of spermatozoa with intact structural and morphology integrity, whereas HSP/GSH increased the observed number of spermatozoa characterized with un-capacitated motility.

Key words: spermatozoa, ram, cryopreservation, reduced-glutathione, homologous-seminal-plasma

HYPOTHESIS AND AIMS

The aims of this research were based on the scientific theories that homologous seminal plasma (HSP) and reduced glutathione (GSH) can exert protective and/or reparative effect on cryopreserved (CP) ram spermatozoa in liquid nitrogen through addition in CP-media prior or following thawing (Barrios et al., 2000; Perez-Pe, et al., 2001). However, to our best knowledge, there is no information of investigating the combined effects of these two components in single CP-media, nor the post-thaw time of occurrence of the aforementioned effects. In the current research, these effects were investigated by assessing spermatozoa structural integrity, morphology, and motility pattern by the factor of post-thaw incubation time, since CP induces significant alterations on these characteristics which can be reversible in physiological temperature conditions (Salamon and Maxwell, 2000; Perez-Pe, et al., 2001). The null hypothesis of this research was as follows: "The combined presence of specific GSH and HSP concentrations, supplemented in CP-media for ram ejaculates, will not cause significant discrepancies in spermatozoa structure, morphology and motility pattern of ovchepolska pramenka ejaculates, compared to control CP-media which are GSH- and HSP-free".

In order to evaluate the null hypothesis, the following research aims were set:

1. To assess the effect of using control cryopreserving media before- or along in the after-cryopreserving period, on structural integrity, morphology and motility pattern of ovchepolska pramenka ejaculates, at 0- and 3-hours post-thaw incubation;

2. To assess the effect of pre-cryopreserving addition of control-, GSH- and/or HSP-cryopreserving media on structural integrity, morphology and motility pattern of ovchepolska pramenka ejaculates, at 0- and 3-hours post-thaw incubation;

3. To assess the effect of post-cryopreserving addition of control-, GSH- and/or HSP-cryopreserving media on structural integrity, morphology and motility pattern of ovchepolska pramenka ejaculates, at 0- and 3-hours post-thaw incubation;

4. To assess the inter-effect of pre- and post-cryopreserving addition of GSH- and/or HSP-cryopreserving media on structural integrity, morphology and motility pattern of ovchepolska pramenka ejaculates, at 0- and 3-hours post-thaw incubation.

MATERIAL AND METHODS

Experimental procedure

Homogenized ejaculate volume (3.0 ml) was divided in five aliquots (Alq 1-5) which were suspended (extended) in one of the ejaculate media, reaching final spermatozoa concentration prior cryopreservation:

1. Alq-1 = E + C (100 x 10^6 cells/ml);
2. Alq-2 = E + C (50 x 10^6 cells/ml);
3. Alq-3 = E + E1 (50 x 10^6 cells/ml);
4. Alq-4 = E + E2 (50 x 10^6 cells/ml);
5. Alq-5 = E + E3 (50 x 10^6 cells/ml),

where, E – fresh ejaculate, C – control media, E1-3 - experimental media: E1 – GSH-5 mM; E2 – HSP-20%; E3 – GSH-5mM+HSP-20%. Each Alq was packed in equal volume samples of 0.5 ml (n=25), and was processed on CT. Immediately following thawing, all samples were placed in incubation conditions (temperature, T=37°C, relative humidity RH=90%), and analyzed in two-time targets: 0- and 3-hours. Samples of Alq1 (b-CT method of extending), prior incubation were divided in four equal volumes and were further extended in the same order of extenders as Alq 2-5 in 1:1 ratio, reaching the same cell concentration. According to the extender, extending method (‘a’ for Alq1 and ‘b’ for Alq 2-5) and incubation time sampling (0- and 3-hours) the following groups have been formed, each with equal sample number (n=25): C-a-0, C-a-3, E1-a-0, E1-a-3, E2-a-0, E2-a-3, E3-a-0, E3-a-3, C-b-0, C-b-3, E1-b-0, E1-b-3, E2-b-0, E2-b-3, E3-b-0 and E3-b-3. Spermatozoa from each sample were assessed for structural integrity, morphology (Hancock-2), and trajectory pattern and velocity (CASA).

Ejaculate sampling

Fresh ejaculates were obtained from ovchepolska pramenka rams (N=10), which were stationed at the Faculty of Veterinary Medicine in Skopje. They have been routinely used for artificial vagina ejaculate collection in aim of acquiring the fresh samples in one day. All procedures including animals for this experiment was in accordance with EU directive

2016/63/EU for animal use in research activities. Collected ejaculates were immediately transferred in standardized laboratory conditions (T=30°C и RH=90%) while being analyzed prior cryopreservation (CP).

Qualitative and quantitative homogenization of fresh ejaculates

Fresh samples qualitative homogenization was achieved by compliance with the following criteria (Bucak et al. 2007):

* Volume ≥ 1.00 mL,
* Density ≥ 4 (gradation 1-5)
* Total motility ≥ 80%
* Membrane integrity ≥ *60%*
* Spermatozoa concentration ≥ 2.5 x10^9/ml.

Quantitative homogenization was achieved by pooling the ejaculates compliant with the previous criteria, adjusting their entry volumes according to its spermatocrit value (Sct):

$$Ve\ (\%) = (S / Si) / \sum_{i=1}^{n} (S / Si) \times 100$$

where, „*Ve*“ is the volume participation of the single ejaculate in the pool (%), „*Si*“ is the spermatocrit value of the single ejaculate, and „*S*“ is the mean of „*Si*“. In this way, the pool contained approximately equal number of spermatozoa from each animal, having similar qualitative characteristics.

Experimental components and ejaculate media

Homologous seminal plasma (HSP)

Seminal plasma was obtained from the same animals and the same day as for the ejaculate collection. The ejaculates for HSP were chilled at 4°C/30 minutes, and were centrifuged in two stages: I – 2000 x g, 4°C, 20 min.; II- 2500 x g, 4°C, 30 min. The supernatants were filtered (0.22 μm), homogenized according their specific weight and cryopreserved (-20°C) until being used (Ghaoui et al., 2007). Prior using, they were thawed at room temperature (21-23°C).

Reduced glutathione

The reduced glutathione solution (GSH – γ-L-Glutamyl-L-cysteinyl-glycine; cell culture tested, ≥98.0%, powder; Sigma Aldrich®), was prepared from pulverized commercial package (307.32 gr/mol). Three volumes of sterile water (SW) were prepared, varying in GSH concentration:

SW1 – (0 mM GSH)

SW2 – 0,040 gr GSH/20,0 ml H_2O (6,25 mM GSH);

SW3 – 0,051 gr GSH/22,0 ml H_2O (7,50 mM GSH).

Ejaculate media

Control extender (C) was prepared from tris-based solution (Cx), GSH- and HSP-free. According to the manufacturer instructions, it was diluted with SW in 1:5 ratio prior use. Extenders were monitored for its final specific weight (S_w=1.039) following addition of SW and experimental components.

Control extender (C) = Cx (32 ml) + SW1 (128 ml) (S_w=1,039);

Experimental extender 1 (E1) = Cx (4 ml) + SW2 (16 ml) (S_w=1,039);

Experimental extender 2 (E2) = C (12 ml) + HSP (3 ml), (S_w=1,039);

Experimental extender 3 (E3) = Cx (4 ml) + SW3 (16 ml) + HSP (4 ml), (S_w=1,039).

Ejaculate pool extension

Ejaculate aliquots from Alq1 were extended with C-media up to 100 x10^6/ml prior freezing and with one of the four media up to 50 x10^6/ml, following thawing (b-CT method). Ejaculate aliquots from Alq2-5 were extended prior freezing up to 50 x10^6/ml, with C, E1-3 in respective order (a-CT method).

Alq1 = C (58.800 ul) + E (1.200 ul) = 60.000 ul (100 x10^6/ml);

Alq2 (C-a) = C (14.850 ul) + E (150 ul) = 15.000 ul (50 x10^6/ml);

Alq3 (E1-a) = E1 (14.850 ul) + E (150 ul) = 15.000 ul (50 x10^6/ml);

Alq4 (E2-a) = E2 (14.850 ul) + E (150 ul) = 15.000 ul (50 x10^6/ml);

Alq5 (E3-a) = E3 (14.850 ul) + E (150 ul) = 15.000 ul (50 x10^6/ml).

Cryopreservation in liquid nitrogen (-196°C)

A 0.5 ml volume of extended ejaculates from each aliquot was packed in plastic straws and cryopreserved in standardized and automated freezing protocol (Ice Cube 15M):

1. -1,6°C/min up to -4°C (10 min);
2. -4°C equilibration (120 min);
3. -5°C/min up to -6°C (2 min);
4. -6°C equilibration (1 min);
5. -4°C/min up to -10°C (1 min);
6. -3,33°C/min up to -20°C (3 min);
7. -60°C/min up to -80°C (1 min);
8. -80°C equilibration (3 min); -20°C/min up to -100 °C (1min);
9. -100°C equilibration (1 min);
10. -10°C/min (2 min);
11. -196°C (6 months).

Post-thaw extension and incubation of samples

Thawing was performed at 38°C for 30 seconds. Alq1 samples were divided in four groups and each was extended with one of the previously described extenders (C, E1-3) in 1:1 ratio.

Alq1 (C-b) = Alq1 (n=30, 0,5 ml) + C (n=30, 0,5 ml);

Alq 1 (E1-b) = Alq1 (n=30, 0,5 ml) + E1 (n=30, 0,5 ml);

Alq1 (E2-b) = Alq1 (n=30, 0,5 ml) + E2 (n=30, 0,5 ml);

Alq1 (E3-b) = Alq1 (n=30, 0,5 ml) + E3 (n=30, 0,5 ml).

Post-thaw incubation of all samples was performed at 37°C and RH=90%, up to 3-hours.

Analytical methods

The assessment of ejaculate volume, density, spermatozoa wave motility and cell concentration were performed only for fresh ejaculates prior pooling. Structural integrity, morphology and CASA parameters were assessed for thawed samples.

Assessment of ejaculate volume

The ejaculate volume was assessed with automated pipette (10-100 uL), by counting the number of aliquots (100 uL) being transferred from the ejaculate collector to a sterile glass tube.

Assessment of ejaculate density

Ejaculate density was assessed according to the following gradation system criteria (WHO, 2010):

(1) – watery consistency (400 – 1.000 x 10^6 spermatozoa/ml);

(2) – pale milky consistency (1.000 – 2.500 x 10^6 spermatozoa/ml);

(3) – watery to creamy consistency (2.500 – 3.500 x 10^6 spermatozoa/ml);

(4) – creamy consistency (3.500 – 4.500 x 10^6 spermatozoa/ml); and

(5) dense creamy consistency (4.000 – 6.000 x 10^6 spermatozoa/ml).

Assessment of spermatozoa wave motility

Spermatozoa wave motility was assessed according to the following gradation system criteria (WHO, 2010):

(1) – very weak (10% motility in spermatozoa population);

(2) – weak (no visible waves, but there is noticeable motility);

(3) – medium (around 45-65% are motile and occasionally generate waves);

(4) – good (good motility with denser waves); and

(5) – excellent (95% of spermatozoa are motile and generate dense and fast waves).

Assessment of ejaculate spermatozoa concentration

Spermatozoa concentration was assessed with hemocytometer (Neubauer chamber), according to WHO laboratory manual for semen analysis (WHO, 2010). Confidence interval (CI) was 95%, calculated according the following formula: $N \pm 19.96 \times \sqrt{N}$, where 'N' is the number of counted cells. Each sample was assessed for CI treshold compliance in two repetitions.

Assessment of spermatozoa structural integrity and morphology

This assessment was performed with supravital staining method, described by Hancock, 1951. Stained ejaculate smears are evaluated under immersion on contrast microscope field (x 1.000), including at least 200 spermatozoa. Structural integrity (viability-VIAB) was assessed according to the protoplasmic- (Pm), acrosome-membrane integrity (Ac) and morphology (Mo). According to their status ('+' unaffected or '–' affected), they were categorized in one of the following VIAB categories:

VIAB1 – Pm+, Ac+, Mo+;

VIAB2 – Pm+, Ac-, Mo+;

VIAB3 – Pm-, Ac+, Mo+;

VIAB4 – Pm-, Ac-, Mo+; and

VIAB5 – Pm-, Ac-, Mo-.

Assessment of spermatozoa motility characteristics

This assessment was performed on computerized semen analyzer (CASA – Hamilton Thorne), according to WHO laboratory manual for semen analysis (WHO, 2010). Each sample was recorded in standardized conditions (chamber height 20,7 nm, 37°C, 500 spermatozoa).

The following CASA settings were used:

- Frames rate (FPS) – 60 Hz

- Number of frames – 30 shots

- Minimum detection threshold – 60

- Minimum size threshold – 5 pix;

- Progressive motility threshold – VAP ≥75 μm/sec and STR ≥80%;

- Static cells threshold – VAP ≤21,9 μm/sec и VSL ≤6 μm/sec.

Spermatozoa counts for each CASA parameter were clustered in three value range categories according to Mortimer and Maxwell, 1999:

- **EL** – head elongation index (spermatozoa head width-to-length ratio) – CASA gradation scale 0-100 % in intervals of 10 %:

EL1 - 0-30%;

EL2 - 31-50%; и

EL3 - 51-100%.

- **VCL** – curvilinear trajectory velocity – CASA gradation scale 0-450 μm/s in intervals of 50 μm/s:

VCL1 - 0-150 μm/s;

VCL2 - 151-250 μm/s; and

VCL3 - 251-450 μm/s.

- **LIN** – trajectory linearity (straight- and curvilinear-trajectory velocity ratio) – CASA gradation scale 10-100 % in intervals of 10 %:

LIN1 - 0-30 %;

LIN2 - 31-50 %; and

LIN3 - 51-100 %.

- **ALH** – amplitude of lateral head displacement – CASA gradation scale 0-18 μm in intervals of 2 μm:

ALH1 - (0-6 μm);

ALH2 (7-12 μm); and

ALH3 (13-30 μm).

The second (middle) clusters were considered as reference value range, the first as lower- and the third as higher-than the reference range Mortimer and Maxwell, 1999. Samples which contained higher proportion of cells in the third cluster were considered to be potentially capacitated (hyperactivated).

Spermatozoa velocity ranges were categorized according to the CASA software criteria described in the previous text.

- **VEL** – Spermatozoa velocity:

- VEL1 - fast (VAP ≥75.0 μm/sec);

- VEL2 – average velocity (VAP ≤75.0 μm/sec и ≥21.9 μm/sec);

- VEL3 - slow (VAP ≤21.9 μm/sec) and;

- VEL4 - static (VAP is 0 μm/sec).

Statistical procedures

Categorical character of acquired data was processed with chi-square test of independence (Pearsons χ^2). Spermatozoa counts in parameter categories were organized in contingency tables with according degrees of freedom (df). Statistical significance of the omnibus χ^2 value was defined according to df, *a priori* calculated alpha (α) and critical χ^2 value in respect of the determined test power (1-β=0,99). The interpretation of the omnibus χ^2 value was performed by calculating adjusted residuals (±z) and relative contribution index (RCI) for each table cell (Beasley, T.M. and Schumacker, R.E., 1995). When $p < \alpha$, RCIs adjusted to average spermatozoa frequency in parameter category, were interpreted as significantly higher- (f_+) or lower- (f_-) than-expected counts. The effects of the factors (extender type, type of extension, incubation time), were described and assessed according to the extent of the group deviation from the respective category sum (percentile value from the total-marginal sum) in the contingency table. This deviation was expressed by the RCI value for the according higher- (f_+) or lower-than-expected value (f_-). Positive effects of the factors were acknowledged to marking RCIs for f_+ in VIAB1, EL1/EL2, VCL1/VCL2, ALH1/ALH2, LIN2/LIN3 and VEL1/VEL2, or marking RCIs for f_- for the remaining categories in each parameter. Negative effects of the factors were attributed to RCIs with inverse contributions on the aforementioned categories. Test sensitivity parameters were estimated with statistics software (G*Power v.3.1),

while χ^2 test was performed in Microsoft Office Professional – Excel 2019. The statistical analysis was performed in seven independent sessions:

1. Descriptive statistics of qualitative and quantitative characteristics of ejaculates in sample pool

2. Descriptive statistics of test sensitivity assessment;

3. Comparison of frequency distributions between samples in C-a and C-b groups at 0-hour incubation;

4. Comparison of frequency distributions between samples in C-a and C-b groups at 3-hour incubation;

5. Comparison of frequency distributions between samples in a-CT groups at 0- and 3-hour incubation;

6. Comparison of frequency distributions between samples in b-CT groups at 0- and 3-hour incubation; и

7. Comparison of frequency distributions between samples in E-groups between a-CT and b-CT, at 0- and 3-hour incubation.

RESULTS AND DISCUSSION

For the purpose of simplifying the presentation of the results in this abbreviated version of the PhD dissertation text, the author has minimized the verbal narration of the numbers. This chapter is restricted to presentation of descriptive statistics and contingency tables. Raw and processed data are available at Harvard Dataverse published under https://doi.org/10.7910/DVN/WLCSMI (full citation of raw data are listed in the "References" section).

Qualitative and quantitative characteristics of ejaculates in sample pool

Fresh ejaculates (table 1) were in compliance with the threshold values of the qualitative parameters (Bucak et al. 2007) which confirmed their homogeneity and has eliminated the animal-subject as factor of variation. By adjusting the entry-volume of each fresh ejaculate, the sample-pool contained approximately equal number of spermatozoa from each animal.

Statistical model sensitivity

Results are presented in table 3.

Comparison of frequency distributions between samples in C-a and C-b groups at 0-hour and 3-hour incubation

The comparison of control groups without or with post-thaw incubation (0- and 3-hour, respectively) (graph 1-12), was performed under the assumption that each category had ≥34 % of spermatozoa and that its χ^2 value has contributed to the omnibus significant χ^2 value by at least 15 % (RCI ≥ 0,15). In this way, the significant discrepancy of certain parameter category was weighed with its contribution to the overall spermatozoa population. The comparison of control groups added prior or following cryopreservation, without incubation (C-a-0 vs C-b-0), revealed significant discrepancy for the static cell number in VEL4 (a-CT < b-CT) category. The comparison of the same groups following 3-hour post-thaw incubation, revealed significant discrepancies in VCL2 (a-CT > b-CT), VCL3 (a-CT < b-CT), ALH2 (a-CT > b-CT), ALH3 (a-CT < b-CT), VEL1 (a-CT > b-CT) and VEL4 (a-CT < b-CT) categories. These findings indicate that control media added in the post-cryopreservation period can significantly increase the observed versus expected frequency of thawed spermatozoa with capacitated-like motility.

Comparison of frequency distributions between samples in a-CT groups at 0- and 3-hour incubation

In the a-CT method samples (table 4-9),

C-a-0 had prominent negative effects for EL, VCL, and ALH, and minor negative for VIAB. Minor positive effect was acknowledged for LIN and moderate for VEL. Sperm population from this group was predominantly characterized by damaged structural integrity, round head morphology, high velocity and deviated trajectory. This motility pattern was classified as hyperactivated.

C-a-3 did not have significant effects for VIAB and EL. High positive effect was acknowledged for LIN and VEL, whereas moderate positive for VCL. Minor negative effect was acknowledged for ALH. In this group, sperm cells predominantly had linear pattern of motility,

without any significant effect to the structural integrity, morphology or head elongation. Hyperactivated motility was not observed in this sperm population.

E1-a-0 had minor positive effect for VIAB, LIN and VEL. Moderate negative effect was acknowledged for EL and VCL, and minor negative for ALH. This group had dominant sperm population which was defined by round sperm heads, intact structure and morphology, and altered linearity of motility. Hyperactivation was present to some extent, but not as high as in C-a-0 group.

E1-a-3 had no significant effect for VIAB, EL and LIN. Minor positive effect was acknowledged for VCL and VEL. Minor negative effect was observed for LIN, and to some extent for VEL. This sperm population was characterized by static or low velocity cells which were not complying with hyperactivated pattern criteria.

E2-a-0 had no significant effect on EL and ALH. Highly positive effect was acknowledged for VIAB, and minor positive for LIN. Minor negative effect was seen for VCL and VEL. This sperm population was defined as structurally and morphologically intact, with no significant deviations of trajectory linearity, but higher frequency of static cells. There was no significant implication for hyperactivated motility in this group.

E2-a-3 had highly negative effect for VIAB and VEL. Moderate positive effect was observed for LIN, and minor positive for EL, VCL and ALH. This group contained sperm population with predominantly damaged acrosome integrity and elongated head shape. Trajectory linearity was not affected and motility was absent since static population was predominant. No hyperactivation pattern of motility was therefore acknowledged.

E3-a-0 had highly positive effects for VIAB, EL and ALH, and minor positive for VCL and VEL. Highly negative effect was acknowledged for ALH. No significant effect was observed for LIN. This sperm population had intact structural and morphological integrity with elongated heads, and unaffected trajectory linearity. Velocities were in the lower ranges, meaning that no hyperactivation pattern was identifiable.

E3-a-3 had highly negative effects for VEL, and moderate for VIAB and ALH. Minor positive effect was acknowledged for EL and LIN. This group predominantly contained population with damaged acrosome integrity, characterized by elongated sperm heads and static motility. Hyperactivated motility was not observable due to the higher frequency of static cells.

Comparison of frequency distributions between samples in b-CT groups at 0- and 3-hour incubation

In the b-CT method samples (table 10-15),

C-b-0 had highly positive effects for VIAB, moderate positive for LIN, and minor positive for VEL. Highly negative effects were acknowledged for EL, VCL and ALH. The sperm population of these samples was predominantly characterized by intact structural and morphological integrity, but round head shapes and deviated linearity of the motility trajectory. Despite this, motility was strongly complying with hyperactivated pattern criteria due to the relatively lower effect on sperm velocities.

C-b-3 had moderate negative effects on VIAB, and VCL, and higher negative for ALH and VEL. Positive effects were observed for LIN. This group did not have significant effect for EL. The sperm population was characterized by affected structural integrity and morphology, hyperactivated motility pattern, but predominantly with static cells.

E1-b-0 had moderate negative effects for VIAB and ALH, and high for VCL. Highly positive effect was observed for LIN. This group had no significant effects for EL and VEL. The sperm population was not profiled as with hyperactivated motility, but was predominantly with affected structural integrity and morphology.

E1-b-3 had moderate negative effect for VIAB and high for VEL. Positive effects were observed for EL, VCL, ALH and LIN. The sperm population was largely static and with affected structural integrity. Sperm heads were with mostly with elongated shapes, but motility was largely affected and static.

E2-b-0 had highly positive effect for VIAB and to some smaller extent for VEL. Negative effects were observed for VCL, ALH and to some extent for LIN. There was no significant effect for EL. The population had intact structural integrity and morphology but its motility was profiled as hyperactivated.

E2-b-3 had highly positive effect for EL, VCL, ALH and LIN, whilst negative for VIAB and VEL. The sperm population predominantly had elliptical head shapes with damaged structural integrity and morphology. The trajectory was linear, but motility itself was largely affected due to the high frequency of static cells.

E3-b-0 had highly positive effect for VIAB and VEL, whereas moderately positive for ALH and LIN. There was moderately small negative effect for VCL and no significant effect for EL. The majority of sperm population was with intact structural integrity and morphology, high velocity and unaffected trajectory linearity.

E3-b-3 had highly negative effect for VIAB, and highly positive for VCL, ALH. Moderately positive effects were observed for LIN and VEL, and no significant for EL. The dominant sperm population was with damaged structural integrity and morphology, but unaffected velocity and trajectory linearity.

Comparison of frequency distributions between samples in E-groups between a-CT and b-CT, at 0- and 3-hour incubation

E1-a-0 had small positive effect on VIAB and LIN. Moderate positive effect was acknowledged for VEL. Highly negative effect was observed for EL, VCL, and ALH. This sperm population was characterized by moderately intact structural integrity and morphology, round head shapes, and highly deviated linearity of trajectory. Motility was dominantly defined with hyperactivated pattern.

E1-a-3 had no significant effect on VIAB and LIN. Small and moderate positive effect was observed for EL and VCL, respectively. Small and moderate negative effects were acknowledged for ALH and VEL. This sperm population was characterized with normal ellipticity of head, small deviation in trajectory linearity and higher proportion of static cells. Hyperactivated pattern was not clearly defined for motile sperm cells.

E2-a-0 had no significant effect on EL and ALH. Small negative effects were seen for VCL, LIN and VEL. High positive effect was observed for VIAB. This sperm population was defined by intact structural integrity and morphology and deviated trajectory of motility. Hyperactivated pattern was recognized for motile cells, despite the high frequency of static cells.

E2-a-3 had small positive effect on EL, VCL and ALH, and moderate for LIN. Moderate and high negative effects were observed for VEL and VIAB, respectively. This sperm population was defined by highly affected structural integrity, normal ellipticity of head shape, unaffected trajectory linearity but higher proportion of static cells.

E3-a-0 had highly positive effects for VIAB, EL and ALH, and smaller positive effects for VCL and VEL. The sperm population was characterized by intact structural integrity, morphology and normal head ellipticity. Trajectory linearity and high velocity of motile spermatozoa was unaltered, therefore, no hyperactivated motility pattern was observed.

E3-a-3 had highly positive effect for VCL and VEL, moderate positive for ALH, and low positive for LIN. Moderate negative effect was acknowledged for VIAB. There was no significant effect for EL. This sperm population was characterized by unaffected trajectory linearity and velocity, but altered structural integrity. No hyperactivated motility patterns were identified for motile sperm cells.

E1-b-0 had no significant effect for EL. High positive effect was acknowledged for LIN. High negative effect was observed for VCL and ALH, and low negative for VIAB and VEL. The sperm population of these samples was characterized by static motility, affected structural integrity and affected trajectory linearity. Hyperactivated pattern of motility was identifiable to some extent for the motile spermatozoa, but were not dominant in the population.

E1-b-3 had no significant effect for EL and ALH. High negative effect was acknowledged for VEL and low negative for VIAB. Low positive effect was observed for VCL and LIN. The dominant sperm population in these samples had damaged structural integrity, without affected trajectory linearity and static motility. No hyperactivated motility pattern was acknowledged for this group.

E2-b-0 had no significant effect for EL and VEL. Moderate positive effect was acknowledged for VIAB. High negative effect was observed for ALH, whereas low negative for VCL and LIN. The dominant sperm population was characterized by unaffected structural integrity and morphology but affected trajectory linearity. Hyperactivated motility pattern was observed.

E2-b-3 had low negative effect for VIAB. High and low negative effect was acknowledged for VEL, respectively. High positive effect was observed for LIN and moderate for EL, VCL and ALH. The sperm population had normal head ellipticity but affected structural integrity. Motility was highly decreased but smaller proportion of motile cells had unaffected trajectory linearity. No hyperactivated motility was acknowledged.

E3-b-0 had moderate positive effect in VIAB and VEL. Moderate negative effect was acknowledged for EL and VCL, whereas small negative for ALH and LIN. This sperm

population was characterized by unaffected structural integrity and velocity, round sperm heads, and affected trajectory linearity. Hyperactivated motility pattern was observable.

E3-b-3 had no significant effect of EL. Moderate positive effects were acknowledged for VCL and ALH, and lower positive for LIN. Low negative effects were observed for VIAB and VEL. The sperm population had affected structural integrity, static motility but unaffected trajectory linearity. No hyperactivated motility pattern was acknowledged of the smaller proportion of motile sperm cells.

General discussion of results

In the a-CT method of sample extending, the highest positive effect on cell structural integrity, morphology, head elongation, velocity and trajectory linearity was acknowledged for E3 extender at 0-hour incubation. By analyzing the differences between the observed and expected distribution frequencies of E1 and E2 extender groups at 0-hour incubation, it was acknowledged that the HSP component in E3-a-0 and E3-b-0 was contributive for the positive effects. This indicated that the individual presence of GSH or HSP in tris-based extender used for diluting samples prior or following CP, was not highly effective for achieving higher-than-expected number of spermatozoa categories which would be defined as structurally, morphologically and trajectory linearity intact. The deduction of these findings was that GSH was contributing the effect of HSP in E3 extender. Other reports have described similar effects of ram HSP protein fraction (RSP-14 and RSP-20) (Perez-Pe et al., 2001; Bernardini et al. 2011). Their ability to adhere on altered regions of the spermatozoa protoplasmic membrane has been used as an explanation of the reparative effect following thawing (Bergeron-Annick et al., 2005). Since these investigations have been focused on the structural integrity of the sperm membranes, our findings are partially compliant. In our results, the HSP groups increased the observed vs expected frequency of static spermatozoa with altered acrosome membranes, meaning that hyperactivation-like events have been initiated. These effects were progressive during the incubation, evident in the 3-hour samples of E2-a-3 group. The hyperactivation or fake-capacitation has been previously described by Cormier and Bailey, 2003, and has been related to increased oxidative radicals or capacitating components of the extending medium. During these events, spermatozoa exhibit hyperactivated pattern of motility, have progressive alterations in structural integrity and eventually exhaust their fertilizing capability (de Graaf, 2007; Ledesma et al. 2005). The control extender at 0-hour in a-CT method has had the highest negative effects on spermatozoa populations, which were

characterized by hyperactivated pattern of motility and affected structural integrity, morphology and trajectory linearity. Following 3-hour incubation, trajectory linearity and sperm velocity was attributed by higher spermatozoa frequency, without significant effect on structural integrity and head elongation. The absence of HSP in the ejaculate extending medium was acknowledged as key factor for decreasing the proportions of capacitation-like events and was used for explanation of the observed spermatozoa frequencies in C-a-3 samples.

In the b-CT method of sample extending, E3 extender group at 0-hour incubation had the highest positive effect on sperm cell structural integrity, morphology, velocity and trajectory linearity. This effect was not integrally observable in all parameters for the control, E1 or E2 extending media at 0-hour incubation. The control and E2 extenders at 0-hour incubation had positively attributed the structural integrity of the sperm population, but have also promoted hyperactivated pattern of motility. At 3-hour incubation, the E3 extender has had continuous positive effect on sperm population trajectory linearity and velocity, but has affected the acrosome stability. Nevertheless, no hyperactivated pattern of motility was acknowledged in these samples. For the remaining extender groups at 3-hour incubation in the b-CT method, the common outcome was the negative effect on velocity and structural integrity. The reparative effects of seminal plasma components added in the post-cryopreservation period has been previously described by Garcia-Lopez et al., 2006; Barrios et al., 2000.

The complementary effects of GSH and HSP has been explained due to the interaction of the seminal plasma protein fractions with its antioxidative components (Garcia-Lopez et al., 2006). The previously mentioned RSP-14 and RSP-20 protein fractions, which are species-specific for ram seminal plasma, have been most highly correlated with the action of several antioxidant enzymes, among which glutathione reductase, which contributes to the activity of GSH (Marti et al., 2007). The lack of positive effect in the GSH containing groups could be explained due to the highly unstable GSH in aerobic conditions (Bilodeau et al., 2000). The capacitation-like events of the HSP containing group spermatozoa (E2 extender), especially at 3-hour incubation of the a-CT method, were explained as a result of increased efflux of membrane phospholipids and cholesterol which are essential for acrosome and protoplasmic membrane stability (Manjunath and Therien, 2002). Our findings are contradicting with reports of the reparative and decapacitating effects of ram seminal plasma on thawed spermatozoa (Vadnais et al., 2005), but the inconsistency of the experimental designs could be the reason for result discrepancies. In another investigation, compliant results have been reported where 20 % ram

seminal plasma supplementation in ejaculate extender has been used (Cormier and Bailey, 2003).

By comparing the two methods of sample extension (a-CT vs b-CT) it was deducible that a-CT had higher positive effects on achieving higher spermatozoa ratios with intact structure, morphology, trajectory linearity and velocity at 0-hour incubation. The observed frequencies of spermatozoa in group categories at 3-hour incubation, were considered as biased due to the significant differences in several parameter categories (VCL, ALH and VEL). The difference in the rate of extension prior cryopreservation could be the reason behind these findings. The additional handling of samples following thawing (extension of samples) is reported as detrimental factor for the spermatozoa cryo-survival rate (Salamon, S. and Maxwell, W. M. 2000).

CONCLUSIONS

- The ovchepolska pramenka fresh ejaculate dilution with control media in the pre- and post-cryopreservation period yields higher-than-expected number of static spermatozoa with affected trajectory linearity, following 3-hour post-thaw incubation;

- When using pre-cryopreservation method (a-CT) of ovchepolska pramenka ejaculate extension, at 0-hour incubation, control media yields higher-than-expected number of spermatozoa with damaged protoplasmic and acrosome membranes, altered head elongation and hyperactivated pattern of motility;

- In respect of the previous context, GSH/HSP containing media yielded higher-than-expected number of spermatozoa with intact protoplasmic and acrosome membranes, intact head elongation, and uncapacitated pattern of motility;

- When using pre- and post-cryopreservation method (b-CT) of ovchepolska pramenka ejaculate extension, at 0-hour incubation, GSH/HSP containing media yielded higher-than-expected number of spermatozoa with intact protoplasmic and acrosome membranes, and uncapacitated pattern of motility;

- At 3-hour incubation, regardless of the ejaculate extension method (a-CT or b-CT), samples were characterized by affected structural integrity (protoplasmic and/or acrosome membranes),

trajectory linearity (progressivity) and/or static motility, and were therefore categorized as unfit for AI;

- HSP/GSH containing cryopreserving media used in the prior- (a-CT) versus the post-cryopreservation period (a-CT), yielded higher-than-observed number of thawed ovchepolska pramenka spermatozoa with intact protoplasmic and acrosome membranes, intact head elongation, and uncapacitated pattern of motility, at 0-hour incubation;

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TABLES AND FIGURES

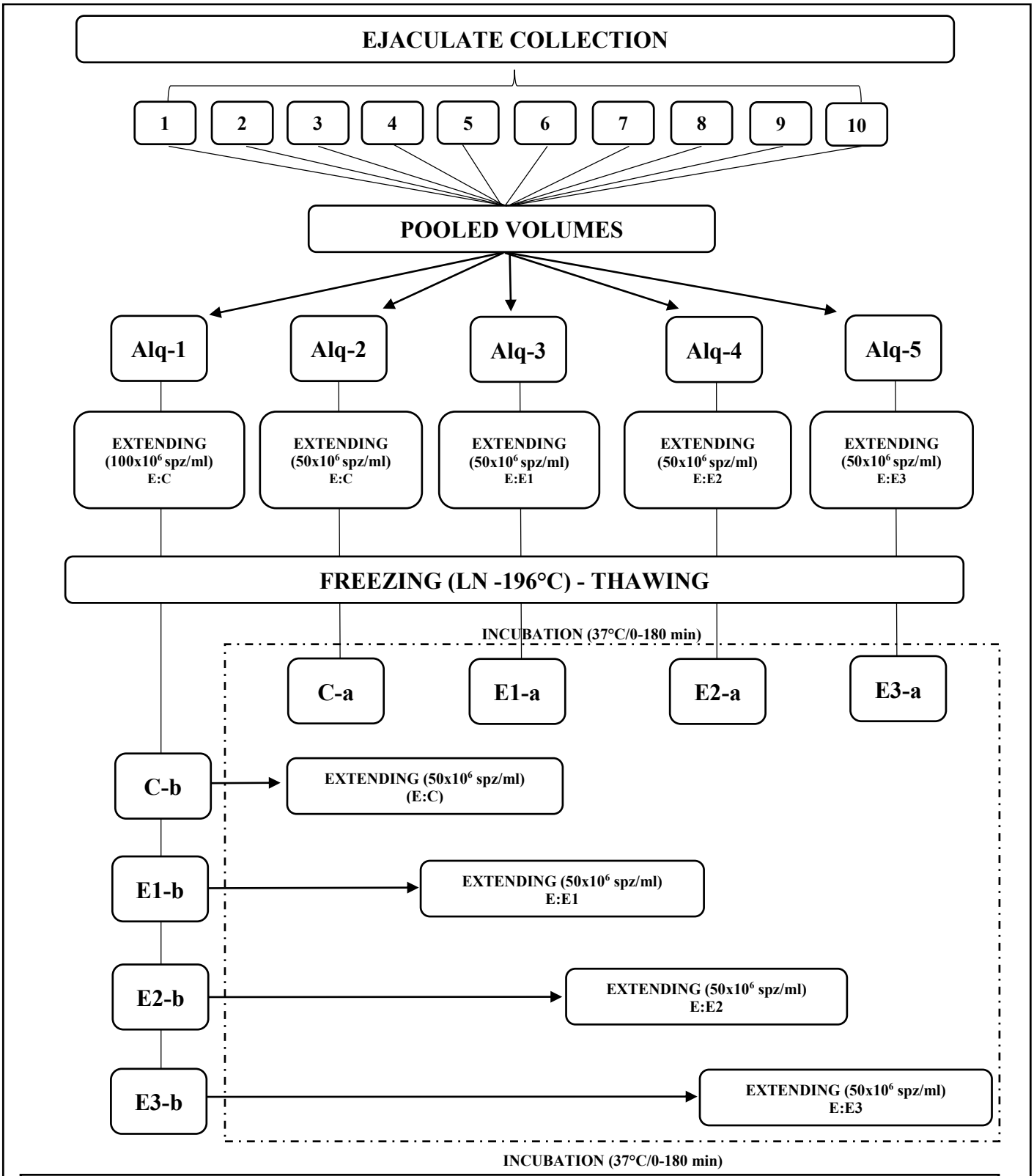


Diagram 1: Diagrammatic presentation of the experimental design

E – Ejaculate; **HSP** – homologous seminal plasma; **Alq 1-5** Aliqoutes; **C** – Control extender; **E1** - Experimental extender 1: C + GSH (5 mM); **E2** Experimental extender 2: C + HSP (20%); **E3** - Experimental extender 3: C + GSH (5 mM) + HSP (20%). Groups **C-a**, **E1-a**, **E2-a**, **E3-a** – Samples treated with C or E prior freezing; groups **C-b**, **E1-b**, **E2-b**, **E3-b** – samples treated with C prior freezing and C or E after thawing.

Table 1. Qualitative and quantitative characteristics of fresh ejaculates

Ram	Vol	Spz	Sct	Den	tMot	VIAB
N	ml	(n x 10^6/ml)	%	(1-5)	%	%
1	1,0	4,32	33	5	97	82,69
2	1,2	2,22	18	4	97	77,78
3	1,3	3,54	37	5	91	83,41
4	2,0	5,88	37	5	93	89,47
5	1,1	3,54	38	5	99	87,02
6	1,1	4,02	32	5	91	82,94
7	1,5	3,60	35	5	93	84,95
8	2,2	3,40	48	5	91	81,55
9	3,0	6,12	43	5	83	93,00
10	2,2	3,40	39	5	92	83,33

Vol – ejaculate volume, Spz – spermatozoa concentration, Sct – spermatocrit, Den – ejaculate density, gradation system 1 to 5 (1-lowest-, 5-highest), tMot – motile spermatozoa (%), VIAB – viable spermatozoa (intact structure and morphology)

Table 2. Quantitative equalization of ejaculate entry volumes in pooled sample

Ram	Vol	H-Sct	H-Spz	Sct	M-Sct/Sct	pVol	pVol
N	(ml)	(cm)	(cm)	%		%	ul
1	1,0	6,0	2,0	33	1,08	10,12	304
2	1,2	6,0	1,1	18	1,95	18,40	552
3	1,3	6,0	2,2	37	0,98	9,20	276
4	2,0	6,0	2,2	37	0,98	9,20	276
5	1,1	6,0	2,3	38	0,93	8,80	264
6	1,1	6,0	2,2	37	0,98	9,20	276
7	1,5	6,0	1,9	32	1,13	10,66	320
8	2,2	6,0	2,1	35	1,02	9,64	289
9	3,0	6,0	2,9	48	0,74	6,98	209
10	2,2	6,0	2,6	43	0,83	7,79	234

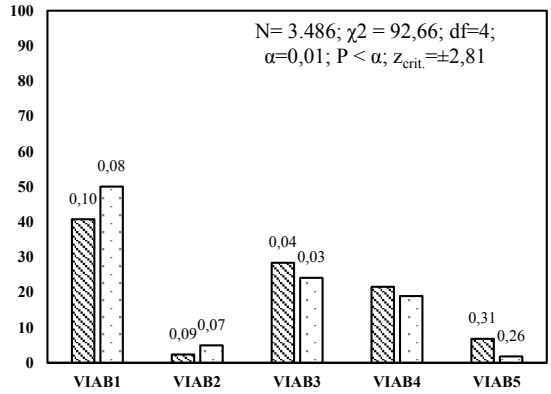
Vol – ejaculate volume, H-Sct – spermatocrit column height, H-Spz – sperm cell column height, Sct – spermatocrit, M-Sct – mean spermatocrit value, pVol – partial ejaculate volume

Table 3. Parameters for test sensitivity of Pearsons χ^2 of independence

		A priori			*Post hoc*	
N	**df**	**λ**	**1-β**	**α**	$z_{crit.}$	χ^2
1	44	76,84	0,99	0,001	± 3,34	78,75
2	33	69,89	0,99	0,001	± 3,28	63,87
3	28	66,34	0,99	0,001	± 3,23	56,89
4	22	50,51	0,99	0,001	± 3,20	40,29
5	21	49,78	0,99	0,01	± 3,16	38,93
6	14	44,02	0,99	0,01	± 3,08	29,14
7	4	31,79	0,99	0,01	± 2,81	13,28
8	3	23,52	0,99	0,05	± 2,73	7,81
9	2	27,41	0,99	0,01	± 2,64	9,21

df – degrees of freedom; λ – acentrality parameter; 1-β – test power; α – *p* value threshold value; $z_{crit.}$ – adjusted residual threshold value; χ^2 –χ^2 treshold value

Graph 1



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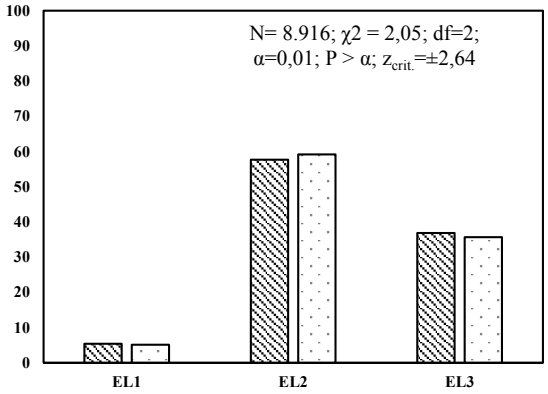

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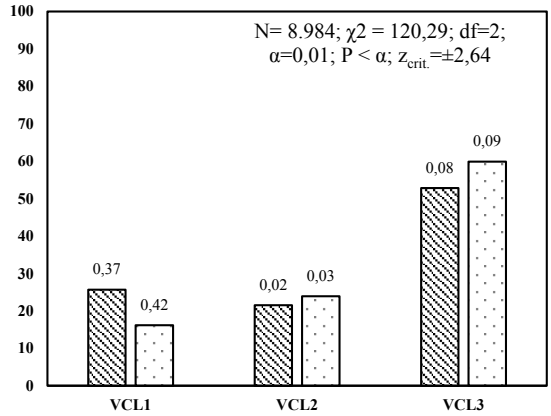

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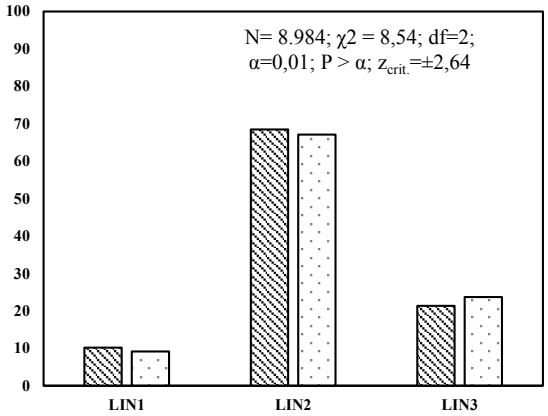

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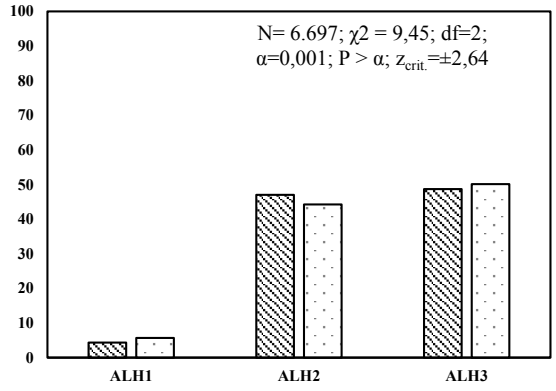

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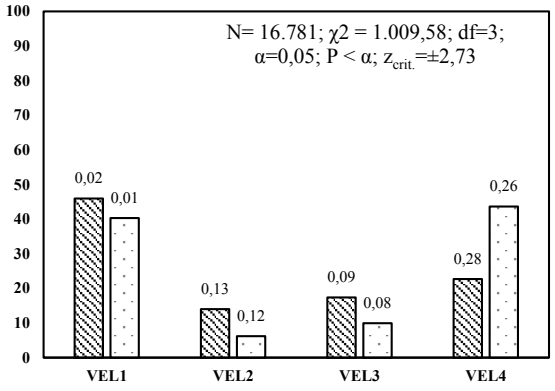
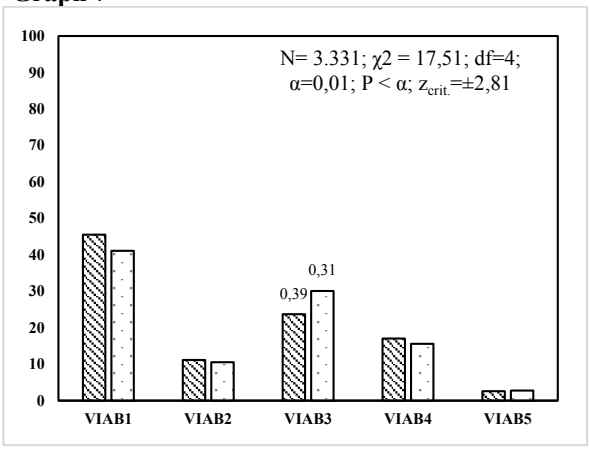


C-a-0 **C-b-0**

Graph 1-6. Spermatozoa distribution frequencies of C-a and C-b samples at 0-hour incubation, in categories according to: 1. structural and morphology integrity (VIAB), 2. spermatozoa head elongation (EL), 3. curvilinear trajectory velocity (VCL), 4. trajectory linearity (LIN), 5. amplitude of lateral head displacement (ALH) and 6. spermatozoa velocity (VEL). N – spermatozoa number; χ^2 –χ^2 test value, α – treshold value for significance of χ^2 value; z – adjusted residual value, $z_{crit.}$ – significance threshold of z value; RCI – relative contribution index (above columns with $z > z_{crit.}$); df – degrees of freedom.

Graph 7



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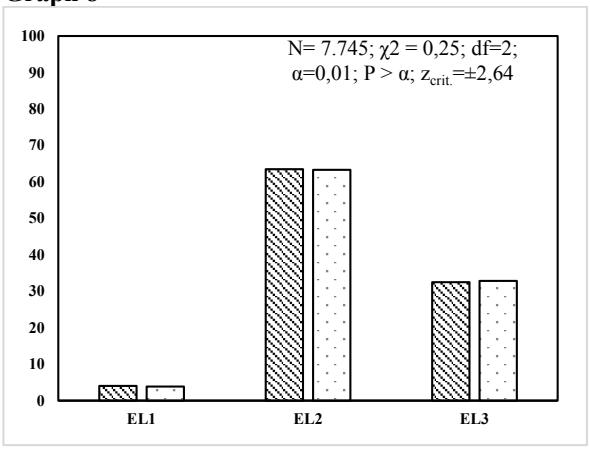
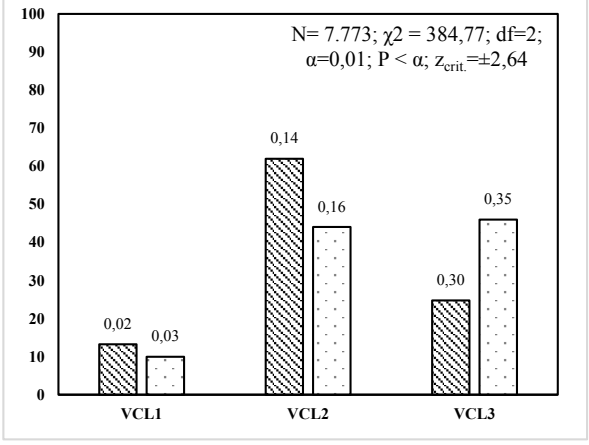


Graph 9



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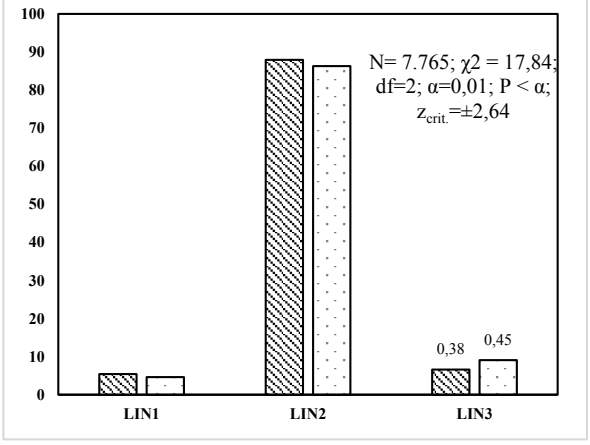

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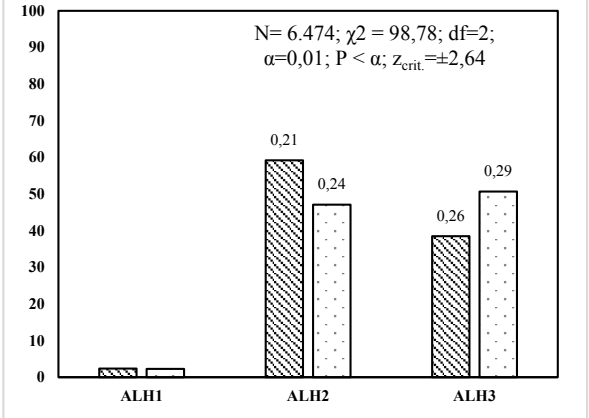
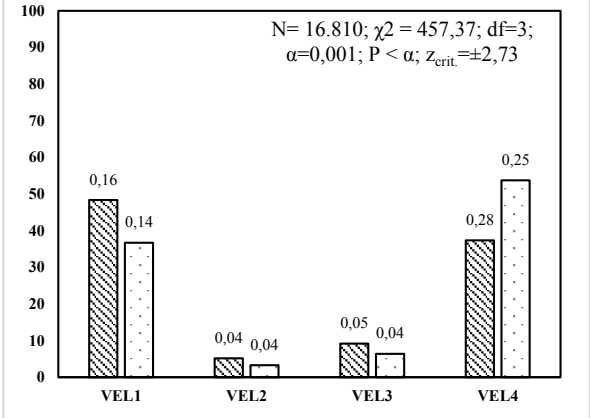


Graph 12



C-a-3 **C-b-3**

Graph 7-12. Spermatozoa distribution frequencies of C-a and C-b samples at 3-hour incubation, in categories according to: 1. structural and morphology integrity (VIAB), 2. spermatozoa head elongation (EL), 3. curvilinear trajectory velocity (VCL), 4. trajectory linearity (LIN), 5. amplitude of lateral head displacement (ALH) and 6. spermatozoa velocity (VEL). N – spermatozoa number; χ^2 –χ^2 test value, α – treshold value for significance of χ^2 value; z – adjusted residual value, $z_{crit.}$ – significance threshold of z value; RCI – relative contribution index (above columns with $z > z_{crit.}$); df – degrees of freedom.

Table 4. Frequency distribution of thawed spermatozoa in structural integrity and morphology categories (VIAB) in a-CT samples at 0- and 3-hour incubation

Groups		C-a-0	C-a-3	E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	Σ row %	χ^2 of independence
Categories											
VIAB1	%	40,78	45,54	46,57	42,27	57,50	21,89	57,16	23,07	**41,51**	N=12.612
	z	-0,63	3,34	4,31	0,65	13,79	-17,57	13,32	-16,21		χ^2=1.750,37
	RCI	0,00	0,00	0,01	0,00	0,06	0,09	0,05	0,08		df=28
VIAB2	%	2,40	11,16	5,17	12,03	3,67	28,88	3,56	24,05	**11,61**	α=0,001
	z	-12,24	-0,57	-8,43	0,56	-10,52	23,79	-10,53	16,83		P < α
	RCI	0,07	0,00	0,03	0,00	0,05	0,25	0,05	0,12		$z_{crit.}$=±3,23
VIAB3	%	28,41	23,69	24,13	23,04	22,42	20,76	23,07	22,83	**23,52**	
	z	4,91	0,17	0,60	-0,47	-1,10	-2,87	-0,44	-0,70		
	RCI	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00		
VIAB4	%	21,59	17,02	21,28	19,67	14,00	25,56	14,45	28,34	**20,36**	
	z	1,30	-3,39	0,96	-0,73	-6,72	5,70	-6,15	8,58		
	RCI	0,00	0,00	0,00	0,00	0,02	0,01	0,02	0,03		
VIAB5	%	6,82	2,59	2,85	2,99	2,41	2,91	1,75	1,71	**3,01**	
	z	9,51	-1,00	-0,39	-0,03	-1,49	-0,26	-3,08	-3,28		
	RCI	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,01		

Groups extended prior cryopreservation ('a'), sampled in two time targets of post-thaw incubation: C-a-0/3, control; E1-a-0/3, GSH-5mM; E2-a-0/3, HSP-20%; E3-a-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to protoplasmic (Pm), acrosome membrane (Ac) and morphology (Mo) status ('+' intact or '-' affected): VIAB1 (Pm+, Ac+, Mo+); VIAB2 (Pm+, Ac-, Mo+); VIAB3 (Pm-, Ac+, Mo+); VIAB4 (Pm-, Ac-, Mo+); and VIAB5 (Pm-, Ac-, Mo-). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 5. Frequency distribution of thawed spermatozoa in head elongation index categories (EL) value range categories in a-CT samples at 0- and 3-hour incubation

Groups		C-a-0	C-a-3	E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	Σ row %	χ^2 of independence
Categories											
EL1	%	5,43	4,05	4,89	6,19	5,38	5,82	5,12	5,52	**5,22**	N=29.135
	z	0,71	-3,66	-1,05	2,79	0,42	1,20	-0,34	0,70		χ^2=194,76
	RCI	0,00	0,06	0,00	0,03	0,00	0,01	0,00	0,00		df=14
EL2	%	57,69	63,47	59,53	60,81	64,41	66,61	67,60	66,23	**62,88**	α=0,01
	z	0,10	0,00	0,04	0,01	0,01	0,02	0,09	0,02		P < α
	RCI	0,07	0,00	0,03	0,00	0,05	0,25	0,05	0,12		$z_{crit.}$=±3,08
EL3	%	36,89	32,48	35,58	33,00	30,21	27,57	27,28	28,26	**31,90**	
	z	8,06	0,86	5,60	1,50	-2,04	-4,10	-7,88	-4,09		
	RCI	0,19	0,00	0,09	0,01	0,01	0,06	0,18	0,05		

Groups extended prior cryopreservation ('a'), sampled in two time targets of post-thaw incubation: C-a-0/3, control; E1-a-0/3, GSH-5mM; E2-a-0/3, HSP-20%; E3-a-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to head elongation index value range (EL): EL1 (0-30%); EL2 (31-50%) и EL3 (51-100%). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 6. Frequency distribution of thawed spermatozoa in curvilinear trajectory velocity (VCL) value range categories in a-CT samples at 0- and 3-hour incubation

Groups		C-a-0	C-a-3	E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	Σ row %	χ^2 of independence
Categories											
VCL1	%	25,68	13,25	20,69	41,73	24,83	38,65	33,31	68,35	**30,79**	N=28.175
	z	-8,43	-26,65	-15,64	15,23	-7,26	7,53	3,75	42,81		χ^2=6.186,63
	RCI	0,01	0,07	0,02	0,02	0,01	0,01	0,00	0,19		df=14
VCL2	%	21,50	61,96	31,30	46,62	35,37	52,67	46,16	30,07	**39,97**	α=0,01
	z	-28,71	31,49	-12,66	8,72	-5,29	11,48	8,70	-10,64		P < α
	RCI	0,07	0,08	0,01	0,01	0,00	0,01	0,01	0,01		$z_{crit.}$=±3,08
VCL3	%	52,82	24,79	48,01	11,64	39,80	8,68	20,53	1,59	**29,24**	
	z	39,47	-6,87	29,51	-24,85	13,06	-20,01	-13,18	-31,99		
	RCI	0,15	0,00	0,08	0,06	0,02	0,04	0,02	0,11		

Groups extended prior cryopreservation ('a'), sampled in two time targets of post-thaw incubation: C-a-0/3, control; E1-a-0/3, GSH-5mM; E2-a-0/3, HSP-20%; E3-a-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to curvilinear trajectory velocity value range (VCL): VCL1 (0-150 μm/s); VCL2 (151-250 μm/s); VCL3 (251-450 μm/s). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 7. Frequency distribution of thawed spermatozoa in trajectory linearity (LIN) value range categories in a-CT samples at 0- and 3-hour incubation

Groups		C-a-0	C-a-3	E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	Σ row %	χ^2 of independence
Categories											
LIN1	%	10,17	5,43	9,20	6,89	7,76	5,24	5,95	4,01	**7,17**	N=28,169
	z	8,83	-4,73	5,61	-0,71	1,27	-3,32	-3,27	-6,46		χ^2=885,41
	RCI	0,07	0,02	0,03	0,00	0,00	0,01	0,01	0,04		df=14
LIN2	%	68,50	87,94	69,07	75,88	69,92	86,41	76,19	82,67	**76,10**	α=0,01
	z	-13,57	19,46	-11,78	-0,32	-8,16	10,70	0,15	8,10		P < α
	RCI	0,04	0,09	0,03	0,00	0,02	0,03	0,00	0,02		$z_{crit.}$=±3,08
LIN3	%	21,33	6,63	21,73	17,23	22,32	8,35	17,86	13,33	**16,73**	
	z	9,40	-18,96	9,58	0,86	8,44	-9,93	2,09	-4,79		
	RCI	0,07	0,29	0,07	0,00	0,06	0,09	0,00	0,02		

Groups extended prior cryopreservation ('a'), sampled in two time targets of post-thaw incubation: C-a-0/3, control; E1-a-0/3, GSH-5mM; E2-a-0/3, HSP-20%; E3-a-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to trajectory linearity value range (LIN): LIN1 (0-30 %); LIN2 (31-50 %); LIN3 (51-100 %). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 8. Frequency distribution of thawed spermatozoa in amplitude of lateral head displacement (ALH) value range categories in a-CT samples at 0- and 3-hour incubation

Groups		C-a-0	C-a-3	E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	Σ row %	χ^2 of independence
Categories											
ALH1	%	4,34	2,35	7,19	6,93	7,20	4,68	6,48	5,03	**5,44**	N=20,009
	z	-3,09	-8,82	4,85	3,26	3,85	-1,27	2,67	-0,67		χ^2=1.009,97
	RCI	0,01	0,06	0,02	0,01	0,01	0,00	0,01	0,00		df=14
ALH2	%	47,00	59,19	53,37	54,78	56,93	69,21	74,97	76,78	**59,51**	α=0,01
	z	-16,21	-0,42	-7,86	-4,81	-2,61	7,44	18,41	12,86		P < α
	RCI	0,09	0,00	0,02	0,01	0,00	0,02	0,12	0,06		$z_{crit.}$=±3,08
ALH3	%	48,66	38,46	39,44	38,30	35,87	26,11	18,55	18,20	**35,04**	
	z	18,15	4,62	5,78	3,40	0,86	-7,05	-20,21	-12,91		
	RCI	0,18	0,01	0,02	0,01	0,00	0,03	0,22	0,10		

Groups extended prior cryopreservation ('a'), sampled in two time targets of post-thaw incubation: C-a-0/3, control; E1-a-0/3, GSH-5mM; E2-a-0/3, HSP-20%; E3-a-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to amplitude of lateral head displacement value range (ALH): ALH1 (0-6 μm); ALH2 (7-12 μm); ALH3 (13-30 μm). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 9. Frequency distribution of thawed spermatozoa in velocity (VEL) value range categories in a-CT samples at 0- and 3-hour incubation

Groups		C-a-0	C-a-3	E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	Σ row %	χ^2 of independence
Categories											
VEL1	%	45,93	48,33	43,77	31,58	29,97	17,53	38,79	16,80	**34,18**	N=62.590
	z	23,76	28,23	19,02	-5,17	-8,34	-32,91	9,30	-34,53		χ^2=5.700,80
	RCI	0,06	0,08	0,04	0,00	0,01	0,11	0,01	0,12		df=21
VEL2	%	14,01	5,15	9,49	14,75	6,84	6,26	11,94	15,61	**10,53**	α=0,01
	z	10,89	-16,59	-3,17	12,97	-11,29	-13,04	4,41	15,60		P < α
	RCI	0,02	0,04	0,00	0,02	0,02	0,02	0,00	0,03		$z_{crit.}$=±3,16
VEL3	%	17,36	9,17	13,28	17,78	17,84	21,02	19,16	12,77	**16,05**	
	z	3,43	-17,74	-7,11	4,43	4,59	12,68	8,12	-8,43		
	RCI	0,00	0,04	0,01	0,00	0,00	0,02	0,01	0,01		
VEL4	%	22,70	37,35	33,46	35,89	45,35	55,20	30,10	54,82	**39,24**	
	z	-32,50	-3,66	-11,14	-6,46	11,75	30,63	-17,90	30,07		
	RCI	0,10	0,00	0,01	0,00	0,01	0,09	0,03	0,08		

Groups extended prior cryopreservation ('a'), sampled in two time targets of post-thaw incubation: C-a-0/3, control; E1-a-0/3, GSH-5mM; E2-a-0/3, HSP-20%; E3-a-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to velocity value range (VEL): VEL1 (≥75.0 μm/s); VEL2 (30,0-74,9 μm/s); VEL3 (0,1-21,9 μm/s), VEL4 (<0,1 μm/s). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 10. Frequency distribution of thawed spermatozoa in structural integrity and morphology categories (VIAB) in b-CT samples at 0- and 3-hour incubation

Groups		C-b-0	C-b-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %	χ^2 of independence
Categories											
VIAB1	%	50,05	41,08	43,60	38,84	48,95	29,67	48,25	33,72	**64,64**	N=16.090
	z	7,92	-0,52	1,91	-2,74	7,09	-11,94	6,57	-7,86		χ^2=842,26
	RCI	0,04	0,00	0,00	0,00	0,03	0,09	0,03	0,04		df=28
VIAB2	%	4,99	10,53	4,79	10,50	5,00	18,26	7,00	16,62	**9,80**	α=0,001
	z	-7,51	1,12	-8,10	1,13	-7,73	13,99	-4,62	11,21		P < α
	RCI	0,05	0,00	0,06	0,00	0,06	0,18	0,02	0,12		$z_{crit.}$=±3,23
VIAB3	%	24,13	30,08	27,93	28,19	26,02	21,73	25,11	19,50	**25,26**	
	z	-1,20	5,09	2,96	3,25	0,84	-3,99	-0,17	-6,48		
	RCI	0,00	0,02	0,01	0,01	0,00	0,01	0,00	0,03		
VIAB4	%	18,98	15,57	21,90	19,07	17,98	27,86	16,77	27,57	**20,83**	
	z	-2,11	-5,93	1,27	-2,08	-3,35	8,53	-4,89	8,12		
	RCI	0,00	0,03	0,00	0,00	0,01	0,06	0,02	0,05		
VIAB5	%	1,84	2,74	1,78	3,40	2,05	2,47	2,87	2,59	**2,47**	
	z	-1,89	0,78	-2,15	2,88	-1,31	0,00	1,27	0,38		
	RCI	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00		

Groups extended following cryopreservation ('b'), sampled in two time targets of post-thaw incubation: C-b-0/3, control; E1-b-0/3, GSH-5mM; E2-b-0/3, HSP-20%; E3-b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to protoplasmic (Pm), acrosome membrane (Ac) and morphology (Mo) status ('+' intact or '-' affected): VIAB1 (Pm+, Ac+, Mo+); VIAB2 (Pm+, Ac-, Mo+); VIAB3 (Pm-, Ac+, Mo+); VIAB4 (Pm-, Ac-, Mo+); and VIAB5 (Pm-, Ac-, Mo-). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 11. Frequency distribution of thawed spermatozoa in head elongation index categories (EL) value range categories in a-CT samples at 0- and 3-hour incubation

Groups		C-b-0	C-b-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %	χ^2 of independence
Categories											
EL1	%	5,14	3,86	4,83	5,24	5,08	4,23	4,10	4,82	**4,66**	N=30.434
	z	1,60	-2,40	0,55	1,76	1,25	-1,24	-1,95	0,52		χ^2=87,12
	RCI	0,02	0,06	0,00	0,03	0,02	0,01	0,04	0,00		df=14
EL2	%	59,17	63,31	62,06	66,05	62,25	66,57	62,18	64,60	**63,14**	α=0,01
	z	-5,72	0,22	-1,48	3,81	-1,15	4,40	-1,47	2,02		P < α
	RCI	0,12	0,00	0,01	0,05	0,01	0,07	0,01	0,02		$z_{crit.}$=±3,08
EL3	%	35,69	32,83	33,11	28,71	32,68	29,20	33,72	30,58	**32,20**	
	z	5,18	0,85	1,28	-4,73	0,63	-3,98	2,40	-2,31		
	RCI	0,18	0,00	0,01	0,15	0,00	0,11	0,04	0,04		

Groups extended following cryopreservation ('b'), sampled in two time targets of post-thaw incubation: C-b-0/3, control; E1-b-0/3, GSH-5mM; E2-b-0/3, HSP-20%; E3-b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to head elongation index value range (EL): EL1 (0-30%); EL2 (31-50%) и EL3 (51-100%). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 12. Frequency distribution of thawed spermatozoa in curvilinear trajectory velocity (VCL) value range categories in b-CT samples at 0- and 3-hour incubation

Groups		C-b-0	C-b-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %	χ^2 of independence
Categories											
VCL1	%	16,19	9,96	16,20	20,15	20,66	20,23	16,99	32,76	**19,10**	N=30.595
	z	-5,15	-14,83	-4,90	1,69	2,48	1,77	-3,98	23,15		χ^2=5.300,43
	RCI	0,00	0,03	0,00	0,00	0,00	0,00	0,00	0,07		df=14
VCL2	%	23,93	44,04	25,24	59,00	32,54	69,07	35,48	57,86	**42,62**	α=0,01
	z	-26,28	1,84	-23,32	21,03	-12,75	33,20	-10,68	20,55		P < α
	RCI	0,06	0,00	0,05	0,04	0,02	0,11	0,01	0,04		$z_{crit.}$=±3,08
VCL3	%	59,88	46,00	58,56	20,85	46,80	10,70	47,53	9,38	**38,28**	
	z	30,90	10,12	27,69	-22,76	10,97	-35,21	14,09	-39,63		
	RCI	0,10	0,01	0,08	0,05	0,01	0,13	0,02	0,16		

Groups extended following cryopreservation ('b'), sampled in two time targets of post-thaw incubation: C-b-0/3, control; E1-b-0/3, GSH-5mM; E2-b-0/3, HSP-20%; E3-b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to curvilinear trajectory velocity value range (VCL): VCL1 (0-150 µm/s); VCL2 (151-250 µm/s); VCL3 (251-450 µm/s). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 13. Frequency distribution of thawed spermatozoa in trajectory linearity (LIN) value range categories in b-CT samples at 0- and 3-hour incubation

Groups		C-b-0	C-b-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %	χ^2 of independence
Categories											
LIN1	%	9,17	4,63	5,79	4,84	7,95	4,36	4,91	4,38	**5,77**	N=30.685
	z	10,15	-3,11	0,06	-2,53	5,78	-3,76	-2,77	-3,96		χ^2=1.229,56
	RCI	0,07	0,01	0,00	0,00	0,02	0,01	0,00	0,01		df=14
LIN2	%	67,11	86,30	67,88	82,73	71,80	89,45	74,16	82,53	**77,33**	α=0,01
	z	-16,96	13,65	-14,98	8,20	-8,17	17,96	-5,71	8,27		P < α
	RCI	0,05	0,03	0,04	0,01	0,01	0,05	0,01	0,01		$z_{crit.}$=±3,08
LIN3	%	23,71	9,07	26,33	12,43	20,25	6,20	20,92	13,09	**16,90**	
	z	12,64	-13,32	16,70	-7,59	5,53	-17,72	8,10	-6,77		
	RCI	0,09	0,11	0,16	0,03	0,02	0,19	0,04	0,03		

Groups extended following cryopreservation ('b'), sampled in two time targets of post-thaw incubation: C-b-0/3, control; E1-b-0/3, GSH-5mM; E2-b-0/3, HSP-20%; E3-b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to trajectory linearity value range (LIN): LIN1 (0-30 %); LIN2 (31-50 %); LIN3 (51-100 %). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 14. Frequency distribution of thawed spermatozoa in amplitude of lateral head displacement (ALH) value range categories in b-CT samples at 0- and 3-hour incubation

Групи		C-b-0	C-b-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %	χ^2 на независност
Категории											
ALH1	%	5,67	2,25	6,84	5,24	6,36	3,52	7,62	4,84	**5,38**	N=24.948
	z	0,80	-8,13	3,88	-0,35	2,37	-4,74	6,77	-1,42		χ^2=1.147,98
	RCI	0,00	0,05	0,01	0,00	0,00	0,02	0,03	0,00		df=14
ALH2	%	44,22	47,07	48,15	59,60	47,81	71,35	54,62	70,33	**55,24**	α=0,01
	z	-13,74	-9,63	-8,53	5,00	-8,17	18,65	-0,84	17,92		P < α
	RCI	0,06	0,03	0,02	0,01	0,02	0,12	0,00	0,11		$z_{crit.}$=±3,08
ALH3	%	50,11	50,68	45,01	35,16	45,83	25,13	37,76	24,84	**39,38**	
	z	13,61	13,56	6,89	-4,93	7,23	-16,79	-2,27	-17,58		
	RCI	0,08	0,09	0,02	0,01	0,02	0,13	0,00	0,14		

Groups extended following cryopreservation ('b'), sampled in two time targets of post-thaw incubation: C-b-0/3, control; E1-b-0/3, GSH-5mM; E2-b-0/3, HSP-20%; E3-b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to amplitude of lateral head displacement value range (ALH): ALH1 (0-6 μm); ALH2 (7-12 μm); ALH3 (13-30 μm). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 15. Frequency distribution of thawed spermatozoa in velocity (VEL) value range categories in b-CT samples at 0- and 3-hour incubation

Groups		C-b-0	C-b-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %	χ^2 of independence
Categories											
VEL1	%	45,93	48,33	43,77	31,58	29,97	17,53	38,79	16,80	**36,59**	N=72.827
	z	23,76	28,23	19,02	-5,17	-8,34	-32,91	9,30	-34,53		χ^2=1.196,64
	RCI	0,06	0,08	0,04	0,00	0,01	0,11	0,01	0,12		df=21
VEL2	%	14,01	5,15	9,49	14,75	6,84	6,26	11,94	15,61	**5,28**	α=0,01
	z	10,89	-16,59	-3,17	12,97	-11,29	-13,04	4,41	15,60		P < α
	RCI	0,02	0,04	0,00	0,02	0,02	0,02	0,00	0,03		$z_{crit.}$=±3,16
VEL3	%	17,36	9,17	13,28	17,78	17,84	21,02	19,16	12,77	**8,94**	
	z	3,43	-17,74	-7,11	4,43	4,59	12,68	8,12	-8,43		
	RCI	0,00	0,04	0,01	0,00	0,00	0,02	0,01	0,01		
VEL4	%	22,70	37,35	33,46	35,89	45,35	55,20	30,10	54,82	**49,20**	
	z	-32,50	-3,66	-11,14	-6,46	11,75	30,63	-17,90	30,07		
	RCI	0,10	0,00	0,01	0,00	0,01	0,09	0,03	0,08		

Groups extended following cryopreservation ('b'), sampled in two time targets of post-thaw incubation: C-b-0/3, control; E1-b-0/3, GSH-5mM; E2-b-0/3, HSP-20%; E3-b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to velocity value range (VEL): VEL1 (≥75.0 μm/s); VEL2 (30,0-74,9 μm/s); VEL3 (0,1-21,9 μm/s), VEL4 (<0,1 μm/s). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 16. Frequency distribution of thawed spermatozoa in structural integrity and morphology categories (VIAB) in a- and b-CT samples at 0- and 3-hour incubation

Groups		E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %
Categories														
VIAB1	%	46,57	42,27	57,50	21,89	57,16	23,07	43,60	38,84	48,95	29,67	48,25	33,72	**40,68**
	z	4,89	1,33	14,13	-16,35	13,67	-15,06	2,80	-1,78	7,90	-10,81	7,40	-6,80	
	RCI	0,01	0,00	0,05	0,07	0,05	0,06	0,00	0,00	0,02	0,03	0,01	0,01	
VIAB2	%	5,17	12,03	3,67	28,88	3,56	24,05	4,79	10,50	5,00	18,26	7,00	16,62	**11,64**
	z	-8,23	0,50	-10,25	22,98	-10,26	16,26	-10,08	-1,69	-9,73	9,95	-6,96	7,44	
	RCI	0,03	0,00	0,04	0,20	0,04	0,10	0,04	0,00	0,03	0,04	0,02	0,02	
VIAB3	%	24,13	23,04	22,42	20,76	23,07	22,83	27,93	28,19	26,02	21,73	25,11	19,50	**23,82**
	z	0,29	-0,75	-1,36	-3,07	-0,72	-0,98	4,55	4,85	2,43	-2,37	1,45	-4,86	
	RCI	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,00	0,00	0,00	0,01	
VIAB4	%	21,28	19,67	14,00	25,56	14,45	28,34	21,90	19,07	17,98	27,86	16,77	27,57	**21,37**
	z	-0,09	-1,70	-7,42	4,38	-6,87	7,14	0,61	-2,65	-3,88	7,65	-5,39	7,26	
	RCI	0,00	0,00	0,02	0,01	0,02	0,02	0,00	0,00	0,00	0,02	0,01	0,02	
VIAB5	%	2,85	2,99	2,41	2,91	1,75	1,71	1,78	3,40	2,05	2,47	2,87	2,59	**2,49**
	z	0,93	1,32	-0,22	1,14	-1,94	-2,09	-2,15	2,76	-1,33	-0,05	1,19	0,32	
	RCI	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	

χ^2 of independence: N=21.885; χ^2=2.185,22; df=44; α=0,001; P < α; $z_{crit.}$=±3,34

Experimental groups extended prior and following cryopreservation ('a' and 'b'), sampled in two time targets of post-thaw incubation: E1-a,b-0/3, GSH-5mM; E2-a,b-0/3, HSP-20%; E3-a,b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to protoplasmic (Pm), acrosome membrane (Ac) and morphology (Mo) status ('+' intact or '-' affected): VIAB1 (Pm+, Ac+, Mo+); VIAB2 (Pm+, Ac-, Mo+); VIAB3 (Pm-, Ac+, Mo+); VIAB4 (Pm-, Ac-, Mo+); and VIAB5 (Pm-, Ac-, Mo-). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 17. Frequency distribution of thawed spermatozoa in head elongation index categories (EL) value range categories in a- and b-CT samples at 0- and 3-hour incubation

Group		E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %
Categories														
EL1	%	4,89	6,19	5,38	5,82	5,12	5,52	4,83	5,24	5,08	4,23	4,10	4,82	**5,03**
	z	-0,45	3,32	0,89	1,58	0,32	1,15	-0,59	0,60	0,14	-2,21	-3,07	-0,62	
	RCI	0,00	0,06	0,00	0,01	0,00	0,01	0,00	0,00	0,00	0,02	0,05	0,00	
EL2	%	59,53	60,81	64,41	66,61	67,60	66,23	62,06	66,05	62,25	66,57	62,18	64,60	**63,91**
	z	-6,30	-4,02	0,58	2,46	5,91	2,49	-2,49	2,77	-2,12	3,37	-2,59	0,94	
	RCI	0,07	0,03	0,00	0,01	0,06	0,01	0,01	0,01	0,01	0,02	0,01	0,00	
EL3	%	35,58	33,00	30,21	27,57	27,28	28,26	33,11	28,71	32,68	29,20	33,72	30,58	**31,06**
	z	6,75	2,61	-1,02	-3,30	-6,28	-3,13	2,86	-3,16	2,13	-2,45	4,14	-0,68	
	RCI	0,16	0,02	0,00	0,04	0,14	0,04	0,03	0,04	0,02	0,02	0,06	0,00	

χ^2 of independence: N=42.908; χ^2=172,74; df=22; α=0,01; P < α; $z_{crit.}$=±3,20

Experimental groups extended prior and following cryopreservation ('a' and 'b'), sampled in two time targets of post-thaw incubation: E1-a,b-0/3, GSH-5mM; E2-a,b-0/3, HSP-20%; E3-a,b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to head elongation index value range (EL): EL1 (0-30%); EL2 (31-50%) и EL3 (51-100%). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 18. Frequency distribution of thawed spermatozoa in curvilinear trajectory velocity (VCL) value range categories in a- and b-CT samples at 0- and 3-hour incubation

Group		E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %
Categories														
VCL1	%	20,69	41,73	24,83	38,65	33,31	68,35	16,20	20,15	20,66	20,23	16,99	32,76	**27,84**
	z	-11,08	19,45	-3,71	10,55	8,17	46,80	-16,91	-10,70	-9,85	-10,37	-17,51	7,17	
	RCI	0,01	0,03	0,00	0,01	0,00	0,16	0,02	0,01	0,01	0,01	0,02	0,00	
VCL2	%	31,30	46,62	35,37	52,67	46,16	30,07	25,24	59,00	32,54	69,07	35,48	57,86	**43,07**
	z	-16,50	4,51	-8,59	8,49	4,19	-13,60	-23,44	20,07	-13,08	32,05	-11,07	19,53	
	RCI	0,01	0,00	0,00	0,00	0,00	0,01	0,03	0,02	0,01	0,06	0,01	0,02	
VCL3	%	48,01	11,64	39,80	8,68	20,53	1,59	58,56	20,85	46,80	10,70	47,53	9,38	**29,09**
	z	28,93	-24,10	13,03	-19,67	-12,63	-31,36	42,24	-11,32	23,98	-24,71	29,35	-28,37	
	RCI	0,06	0,04	0,01	0,03	0,01	0,07	0,12	0,01	0,04	0,04	0,06	0,06	

χ^2 of independence: N=42,013; χ^2=9.395,95; df=22; α=0,01; P < α; $z_{crit.}$=±3,20

Experimental groups extended prior and following cryopreservation ('a' and 'b'), sampled in two time targets of post-thaw incubation: E1-a,b-0/3, GSH-5mM; E2-a,b-0/3, HSP-20%; E3-a,b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to curvilinear trajectory velocity value range (VCL): VCL1 (0-150 μm/s); VCL2 (151-250 μm/s); VCL3 (251-450 μm/s). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 19. Frequency distribution of thawed spermatozoa in trajectory linearity (LIN) value range categories in a- and b-CT samples at 0- and 3-hour incubation

Group		E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %
Categories														
LIN1	%	9,20	6,89	7,76	5,24	5,95	4,01	5,79	4,84	7,95	4,36	4,91	4,38	**6,00**
	z	9,36	2,36	4,09	-1,40	-0,15	-4,35	-0,57	-3,05	4,99	-4,22	-3,37	-4,45	
	RCI	0,06	0,00	0,01	0,00	0,00	0,01	0,00	0,01	0,02	0,01	0,01	0,01	
LIN2	%	69,07	75,88	69,92	86,41	76,19	82,67	67,88	82,73	71,80	89,45	74,16	82,53	**76,71**
	z	-12,55	-1,23	-8,88	10,04	-0,82	7,30	-13,60	8,90	-7,06	18,39	-4,44	8,99	
	RCI	0,03	0,00	0,01	0,02	0,00	0,01	0,03	0,01	0,01	0,06	0,00	0,01	
LIN3	%	21,73	17,23	22,32	8,35	17,86	13,33	26,33	12,43	20,25	6,20	20,92	13,09	**17,29**
	z	8,15	-0,10	7,36	-10,34	1,02	-5,43	15,56	-8,04	4,76	-17,90	7,08	-7,26	
	RCI	0,04	0,00	0,03	0,07	0,00	0,02	0,15	0,04	0,01	0,20	0,03	0,03	

χ^2 of independence: N=42.105; χ^2=1.195,46; df=22; α=0,01; P < α; $z_{crit.}$=±3,20

Experimental groups extended prior and following cryopreservation ('a' and 'b'), sampled in two time targets of post-thaw incubation: E1-a,b-0/3, GSH-5mM; E2-a,b-0/3, HSP-20%; E3-a,b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to trajectory linearity value range (LIN): LIN1 (0-30 %); LIN2 (31-50 %); LIN3 (51-100 %). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 20. Frequency distribution of thawed spermatozoa in amplitude of lateral head displacement (ALH) value range categories in a- and b-CT samples at 0- and 3-hour incubation

Group		E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %
Categories														
ALH1	%	7,19	6,93	7,20	4,68	6,48	5,03	6,84	5,24	6,36	3,52	7,62	4,84	**6,13**
	z	2,70	1,63	2,18	-2,24	0,84	-1,65	1,76	-2,08	0,52	-6,16	4,16	-3,13	
	RCI	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,01	0,01	
ALH2	%	53,37	54,78	56,93	69,21	74,97	76,78	48,15	59,60	47,81	71,35	54,62	70,33	**60,30**
	z	-8,59	-5,50	-3,35	6,77	16,99	12,16	-14,64	-0,81	-13,80	12,82	-7,77	11,92	
	RCI	0,02	0,01	0,00	0,01	0,08	0,04	0,06	0,00	0,05	0,04	0,02	0,04	
ALH3	%	39,44	38,30	35,87	26,11	18,55	18,20	45,01	35,16	45,83	25,13	37,76	24,84	**33,57**
	z	7,53	4,88	2,36	-5,87	-18,03	-11,76	14,27	1,90	14,03	-10,15	5,93	-10,76	
	RCI	0,03	0,01	0,00	0,02	0,15	0,07	0,09	0,00	0,09	0,05	0,02	0,05	

χ^2 of independence: N=31.786; χ^2=1.332,75; df=22; α=0,01; P < α; $z_{crit.}$=±3,20

Experimental groups extended prior and following cryopreservation ('a' and 'b'), sampled in two time targets of post-thaw incubation: E1-a,b-0/3, GSH-5mM; E2-a,b-0/3, HSP-20%; E3-a,b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to amplitude of lateral head displacement value range (ALH): ALH1 (0-6 μm); ALH2 (7-12 μm); ALH3 (13-30 μm). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.

Table 21. Frequency distribution of thawed spermatozoa in velocity (VEL) value range categories in a- and b-CT samples at 0- and 3-hour incubation

Group		E1-a-0	E1-a-3	E2-a-0	E2-a-3	E3-a-0	E3-a-3	E1-b-0	E1-b-3	E2-b-0	E2-b-3	E3-b-0	E3-b-3	Σ row %
Categories														
VEL1	%	43,77	31,58	29,97	17,53	38,79	16,80	36,43	33,40	32,22	33,85	45,24	34,76	**33,14**
	z	20,69	-3,05	-6,17	-30,29	11,17	-31,86	7,05	0,55	-1,95	1,49	25,73	3,50	
	RCI	0,04	0,00	0,00	0,08	0,01	0,09	0,00	0,00	0,00	0,00	0,06	0,00	
VEL2	%	9,49	14,75	6,84	6,26	11,94	15,61	5,11	5,27	6,45	3,66	5,70	6,57	**7,93**
	z	5,31	23,17	-3,68	-5,64	13,83	26,09	-10,52	-9,91	-5,41	-15,78	-8,27	-5,09	
	RCI	0,00	0,06	0,00	0,00	0,02	0,08	0,01	0,01	0,00	0,03	0,01	0,00	
VEL3	%	13,28	17,78	17,84	21,02	19,16	12,77	9,55	6,85	12,52	7,81	10,27	8,33	**12,77**
	z	1,39	13,75	13,90	22,55	17,83	-0,02	-9,76	-17,88	-0,77	-14,87	-7,49	-13,53	
	RCI	0,00	0,02	0,02	0,06	0,04	0,00	0,01	0,04	0,00	0,02	0,01	0,02	
VEL4	%	33,46	35,89	45,35	55,20	30,10	54,82	48,90	54,48	48,81	54,68	38,79	50,33	**46,16**
	z	-23,34	-18,88	-1,49	16,56	-29,98	15,95	5,57	16,82	5,29	17,10	-14,80	8,52	
	RCI	0,04	0,03	0,00	0,02	0,06	0,02	0,00	0,02	0,00	0,02	0,02	0,01	

χ^2 of independence: N=101.826; χ^2=7.037,72; df=33; α=0,001; P < α; $z_{crit.}$=±3,28

Experimental groups extended prior and following cryopreservation ('a' and 'b'), sampled in two time targets of post-thaw incubation: E1-a,b-0/3, GSH-5mM; E2-a,b-0/3, HSP-20%; E3-a,b-0/3, GSH-5mM+HSP-20%, where GSH is reduced glutathione, HSP is homologous seminal plasma. Spermatozoa categories according to velocity value range (VEL): VEL1 (≥75.0 μm/s); VEL2 (30,0-74,9 μm/s); VEL3 (0,1-21,9 μm/s), VEL4 (<0,1 μm/s). N – total number of analyzed spermatozoa; χ^2 –χ^2 value; α – *p* value threshold value; z – adjusted residual value; $z_{crit.}$ – adjusted residual threshold value; RCI – relative contribution index; df – degrees of freedom.