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THE EFFECT OF EXTENDING RAM SPERM BEFORE AND AFTER CRYOPRESERVATION ON THEIR VIABILITY AND VELOCITY

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ABSTRACT

The current study aimed to assess the effect of adding cryopreserving extender (soy-bean lecithin-SBLE), reduced glutathione (GSH), and seminal plasma (SP) before and after thawing on viability and velocity of cryopreserved ram sperm in liquid nitrogen. Fresh ejaculates (Ovchepolska pramenka rams, n=10) were collected and pooled. One portion was extended up to 50 million/ml with SBLE (control C-a), SBLE and GSH 5 mM (E1-a), SBLE and SP 20 vol% (E2-a), and SBLE, GSH 5 mM and SP 20 vol% (E3-a), respectively. The second portion was extended with SBLE to 100 million/ml. Both portions were cryopreserved in liquid nitrogen. Following thawing, the second portion was extended in the same manner to 50 million/ml and was separated into C-b, E1-b, E2-b, and E3-b, respectively. Each group was sampled in ten replicates immediately following thawing. Thawed samples were analyzed for viability (Hancock-2 stain), and velocity (Hamilton Thorne, USA). Each sample included at least 200 cells and the results were expressed in percent values (mean±SEM). Normality (Kolmogorov) and variance comparison (factorial-ANOVA) were performed in Statistica 8 with a significance level $p<0.05$. E2-a (57.58% ±2.40) and E3-a (56.94% ±1.85) yielded significantly higher viability compared to the C-a (40.73 ±1.53). There were no significant differences between C-b (50.00% ±2.33), E1-b (43.61% ±1.37), E2-b (49.16% ±1.50), and E3-b (48.50% ±1.85). In conclusion, the addition of SBLE, GSH, and SP prior vs after cryopreservation has a significant effect on thawed ram sperm viability and velocity.

Key words: spermatozoa, seminal plasma, reduced glutathione, liquid nitrogen, CASA

INTRODUCTION

Ram sperm cells are highly susceptible to cryoinjury during cryopreservation in liquid nitrogen (Pini et al., 2018; Barbas & Mascarenhas, 2008). Sperm plasma and acrosome membranes are the most vulnerable structures and can trigger capacitation-like changes rendering the sperm prematurely capable of fertilization hence shortening the time for post-cryopreservation handling (Bailey et al., 2000). Mature sperm cells are transcriptionally and translationally silent and are highly reliant on extracellular compounds to retain their viability, motility, and fertilizing ability. The seminal plasma-to-sperm ratio is therefore highly important for the normal function of sperm cells (Leahy et al., 2018). Specific protein fractions in the seminal plasma (SP) are regulating the sperm response to stimuli, its transition to different

metabolic states, the integrity of the plasma and acrosome membranes, and are preventing premature capacitation (Tseng et al., 2013). These compounds are either not included or they are unable to be synthetically replicated in the cryopreservation media (CM). Thus, the dilution of fresh ejaculates with cryopreserving media lowers the SP-proteins-to-sperm reducing its protective effect which is known as the 'dilution effect' (Leahy et al., 2018).

Up to recently, cryo-science was mostly focused on testing the supplementation of various synthetic and biological components in CM but it seemed that the complex composition of SP was impossible to be replicated in a way that would mimic the physiological extracellular environment of the sperm. Therefore, there was an increasing interest in investigating the effects of adding SP to sperm which yielded some promising results (Muiño-Blanco et al., 2008; Leahy & Gadella, 2011; Leahy & de Graaf, 2012). Specific proteins were identified with the ability to bind and stabilize the plasma membrane (Barrios et al., 2000; Muiño-Blanco et al., 2008). The SP was also attributed to the antioxidant effect on sperm cells (Marti et al., 2007). However, the high intra-species variability of SP composition, the numerous factors that might affect the sperm response to its components, and variations in experimental protocols might be the cause for reporting non-existing (Morrier et al., 2003) or even deleterious effects on ram sperm (de Graaf et al., 2007). The use of antioxidants as CM supplements was also investigated as a possible method for alleviating the negative effects of peroxidation on sperm motility and viability.

Among numerous antioxidants, the reduced glutathione (GSH) showed some promising results in rams increasing the activity of glutathione peroxidase and superoxide dismutase (Silva et al., 2011; Zeitoun & Al-Damegh, 2014). Other papers did not report a significant effect of GSH most likely due to different experimental protocols and individual variations (Camara et al., 2011, Anel-Lopez et al., 2012).

To the best of our knowledge, the effect of supplementing SP and GSH to CP was reported individually, solely in the pre-freezing or post-thawing period of cryopreservation, and usually without consideration of the treatment time. This study hypothesized that the combined treatment with these supplements in the post-thawing phase following appropriate incubation time would yield a positive effect on ram sperm viability and motility compared to the control CP. Due to the reported inconsistent effects of these components, the current study aimed to investigate the effect of individual and combined treatment of cryopreserving ram sperm with GSH and SP, the effect of pre-freezing and post-thawing treatment, and the effect of post-treatment time on cryopreserving ram sperm.

MATERIAL AND METHODS

Animals

Ejaculates were collected from ten rams which were housed on the premises of the Faculty of veterinary medicine – Skopje, Ss. Cyril and Methodius University in Skopje. The animals were kept in standardized conditions and were fed with a standard feed at least 2 months before the semen collection. The ram age was between 3 and 8 years. The animals were accustomed to the semen collection method and were confirmed with good health status and positive spermatogram. The procedure was conducted in accordance with the EU directive 2016/63/EU on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Ejaculates

The fresh ejaculates were checked for minimum quality criteria: volume ≥1 mL, density and consistency ≥4 (grading system 0-5), motility ≥80%, viability ≥60%, cell concentration $2.5x10^9$/mL) (Bucak et al., 2007). Pooling was performed by adjusting the ejaculate entry-volume according to its sperm-to-SP ratio (spermatocrit) thus equalizing sperm concentration from each ejaculate (Nikolovski et al., 2019).

Reagents and extenders preparation

The SP was collected from another batch of fresh ejaculates which were centrifuged at 2,000 x g, 4°C for 20 min, and then at 2,500 x g, 4°C for 30 min (El-Hajj Ghaoui et al., 2007). The extracted SP was pooled and frozen at -20°C until being used.

The GSH was provided in commercial packaging (γ-L-Glutamyl-L-cysteinyl-glycine; cell culture tested, ≥98.0%, powder; Sigma Aldrich®) and was used to prepare appropriate solutions.

The control (C) was a soy-bean lecithin-based extender. Three experimental extenders were prepared: E1 = C + GSH (5mM), E2 = C + SP (vol. 20%), and E3 = C + GSH (5 mM) + SP (vol. 20%).

Cryopreservation, thawing, and post-thaw incubation

The samples were packed in plastic straws (0.5 mL) and were automatically frozen in liquid nitrogen according to a freezing protocol for ram sperm including 11 stages (Ice Cube 15M) (García-Álvarez et al., 2009): 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 (2 weeks). The frozen straws were kept in liquid nitrogen (-196°C for 2 weeks). The thawing was performed in a water bath (38°C for 30 s) and the samples were transferred to glass tubes which were incubated at 37°C and 90% air humidity for up to 3 hours.

Sperm viability and motility assessment

Viability was assessed according to the plasma (Pm) and acrosome membrane (Am) integrity, and morphology (Mo) by using the Hancock-2 staining method (Nikolovski et al., 2019). A 5 uL drop of the prewarmed (37°C) stain was mixed with a 5 uL drop of sample and was smeared. At least 200 cells for each sample were evaluated for plasma and acrosome membrane integrity, and morphology. According to the status (unaffected '+'/affected '-'), the cells were categorized as follows: 'viable' if Pm+, Ac+, Mo+; 'damaged acrosome' if Pm+, Ac-, Mo+; 'damaged plasma membrane' if Pm-, Ac+, Mo+; and 'pathological' if Pm-, Ac-, Mo- (Nikolovski et al., 2019). The values were summarized and presented in percent numeric format.
Motility was assessed on CASA (Hamilton Thorne v.12) with the following settings: frames per second– 60 Hz, number of frames – x30; minimum contrast threshold for cell detection – 60; minimum cell size threshold - 5 pix; high-velocity cells threshold – VAP ≥75 u/s; static cells threshold – VAP ≤21.9 u/s and VSL ≤6 u/s. Each sample (6.5 uL) was placed on a microscope slide (25 mm x 75 mm) and covered with a cover slip (18 mm x 18 mm) creating a chamber depth of 20 um. (WHO, 2010). Scanning was performed on the same start-end position of each slide (5-10 mm), including 500 ±50 cells for each sample. According to the cut-off values, the cells were categorized as 'high-velocity', 'motile', and 'progressively motile' cells (Nikolovski et al., 2019). The values were summarized and presented in percent numeric format.

Experimental design

The pooled fresh ejaculates were divided into 5 aliquots. The first aliquot was extended with the control medium up to half of the final sperm concentration ($50x10^6$/mL), whereas the second, third, fourth, and fifth aliquot with C, E1, E2, and E3 extenders, respectively up to the final sperm concentration. The samples were then frozen in liquid nitrogen according to the previously described method. Following thawing, the first aliquot was divided into four sub-aliquots and they were extended in the same manner as aliquots 2-5 in the previous description. Ultimately, 8 groups of samples were formed C, E1, E2, and E3 marked with 'a' (pre-freezing treatment) and 'b' (post-thawing treatment) extended to the final sperm concentration before or after cryopreservation, respectively. The samples were placed in incubation conditions and were sampled at 0 and 3 hours. Each group was sampled in 20 replicates.

Statistical analysis

The acquired data contained numerical percent values for each replicate of the corresponding group which was presented as mean ±SE. The normality was checked with the Kolmogorov-Smirnov test and if a normal distribution was confirmed, factorial ANOVA was employed for assessment of the group and type of treatment effects, and repeated-measures ANOVA for assessment of incubation-time effect (IBM SPSS Statistics, IBM®, USA). Alternatively, the Kruskal-Wallis test was employed if a non-normal distribution was observed between the compared groups. Power analysis was employed to confirm that the employed tests had statistical power ≥0.95 (G*Power v.3.1.9.2, Franz Faul, Kiel University, Germany). Significant differences were considered if $p<0.05$.

RESULTS AND DISCUSSION

Viability

In the viable cell assessment of the pre-freezing treatment E2-a-0 (57.58% ±2.40) and E3-a-0 (56.96% ±1.75) were significantly higher than C-a-0 (40.73% ±1.53). No significant difference was observed between the E1-a-0 (43.61% ±1.37) and C-a-0. In the 3-hour samples, E2-a-3 (21.85% ±1.36) and E3-a-3 (23.44% ±2.30) were significantly lower compared to the C-a-3 (45.40% ±2.72) and E1-a-3 (41.91% ±1.85). Significantly higher values were observed for E2-a-0 and E3-a-0 compared to the 3-hour counterpart samples. In the post-thawing treatment E1-b-0 (43.61 ±1.37) had a significantly lower value compared to the C-b-0 (50% ±2.33). E2-b-0 (49.16% ±1.50) and E3-b-0 (48.50% ±1.85) were not significantly different from the other groups. In the 3-hour samples of the same treatment, E2-b-3 (29.33% ±1.79) was significantly lower than the C-b-3 (40.91% ±2.23) and E1-b-3 (39.15% ±2.48). The E3-b-3 (34.04 ±2.75) group was significantly lower compared to C-b-3. The samples in C-b-0, E2-b-0, and E3-b-0 were significantly higher compared to the corresponding 3-hour samples. Additional information can be found in Figure 1-A.

In the damaged acrosome cell assessment for 0-hour samples, no significant differences were observed in the groups of the pre-freezing and post-thawing treatments. The E3-a-0 (3.59% ±0.56) showed a significantly lower value compared to the E3-b-0 (6.99% ±1.22). However, significantly higher values were observed for all 3-hour samples compared to the 0-hour samples. In the 3-hour samples, E2-a-3 (29.25 ±2.37) and E3-a-3 (24.03 ±1) were significantly higher than C-a-3 (10.80% ±1.32) and E1-a-3 (11.65% ±1.71), but also higher than E2-b-3 (18.33% ±1.72) and E3-b-3 (16.63% ±1.15). Additional information can be found in Figure 1-B.

In the damaged plasma membrane assessment for 0-hour samples in the pre-freezing treatment, C-a-0 (28.28% ±1.79) was significantly higher than E2-a-0 (22.28% ±1.38) and E3-a-0 (23.17% ±0.97). No significant differences were observed between the 0- and 3- hour samples nor between the corresponding 0-hour samples from the post-thawing treatment. The 3-hour samples of the pre-freezing treatment and the 0-hour samples of the post-thawing treatment were not significantly different. C-b-3 (30.17 ±2.33) and E1-b-3 (27.80 ±2.14) were significantly higher compared to E2-b-3 (21.83% ±1.25) and E3-b-3 (19.45% ±1.26) but also significantly higher compared to the C-a-3 (23.88% ±2.12) and E1-a-3 (23.13% ±1.51) samples from the pre-freezing treatment. The C-b 3-hour samples were significantly higher, whereas the E3-b 3-hour samples were significantly lower compared to the corresponding 0-hour samples in C-b-0 (24.26% ±1.84) and E3-b-0 (24.99% ±1.91). Additional information can be found in Figure 1-C.

In the pathological cells assessment, C-a-0 (6.86% ±1.04) was significantly higher compared to the E1-a-0 (2.80% ±0.58), E2-a-0 (2.41% ±0.39), and E3-a-0 (1.73% ±0.24) but also compared to the corresponding C-a-3 (2.75% ±0.50) from the same treatment. C-b-0 (1.83% ±0.40) was significantly lower compared to the C-a-0 in the 0-hour samples. E1-b-0 (1.80% ±0.36) was significantly lower compared to the E1-b-3 (3.34% ±0.60) in the post-thawing treatment. Additional information can be found in Figure 1-D.

These findings indicate that the pre-freezing treatment with SP and GSH/SP immediately following thawing yielded high sperm viability, lower incidence of plasma and acrosome membrane damage, and lower incidence of affected morphology compared to the control. However, this effect dissipated following 3-hours of incubation due to the increased acrosome damage. The affected acrosome could be explained by the increased capacitation of viable cells which was not present in the control and the GSH-treated samples due to the low viability count. Similarly, the SP and GSH/SP-treated samples in the post-freezing treatment showed similar behavior in the viability and damaged acrosome following 3-hour incubation. Despite the higher incidence of sperm with damaged acrosome, interestingly the incidence of sperm with damaged plasma and intact acrosome membranes was lower compared to the 0-hour samples. This might indicate that the effect of SP and GSH/SP post-freezing treatment could be correlated with incubation time which was not the case for the pre-freezing counterpart samples.

It could be proposed that the effect of SP and GSH/SP in the pre-freezing treatment caused a higher capacitation dynamic in viable sperm which rendered the semen ready for immediate use following thawing in *in-vitro* conditions. Its use for *in-vivo* conditions might not yield fertility rates as would be expected and previously reported in other studies (Barrios et al., 2000; El-Hajj Ghaoui et al., 2007; Pérez-Pé et al., 2001). Additionally, even though the post-thawing treatment did not yield significantly higher viability for the SP and GSH/SP treatment, it did indicate that it could have a beneficial effect on plasma membrane integrity following 3-hours of incubation which could be explained by the findings of several reports on the reparative effects of specific protein fractions in the seminal plasma on the sperm plasma membrane (Barrios et al., 2000; Muiño-Blanco et al., 2008).

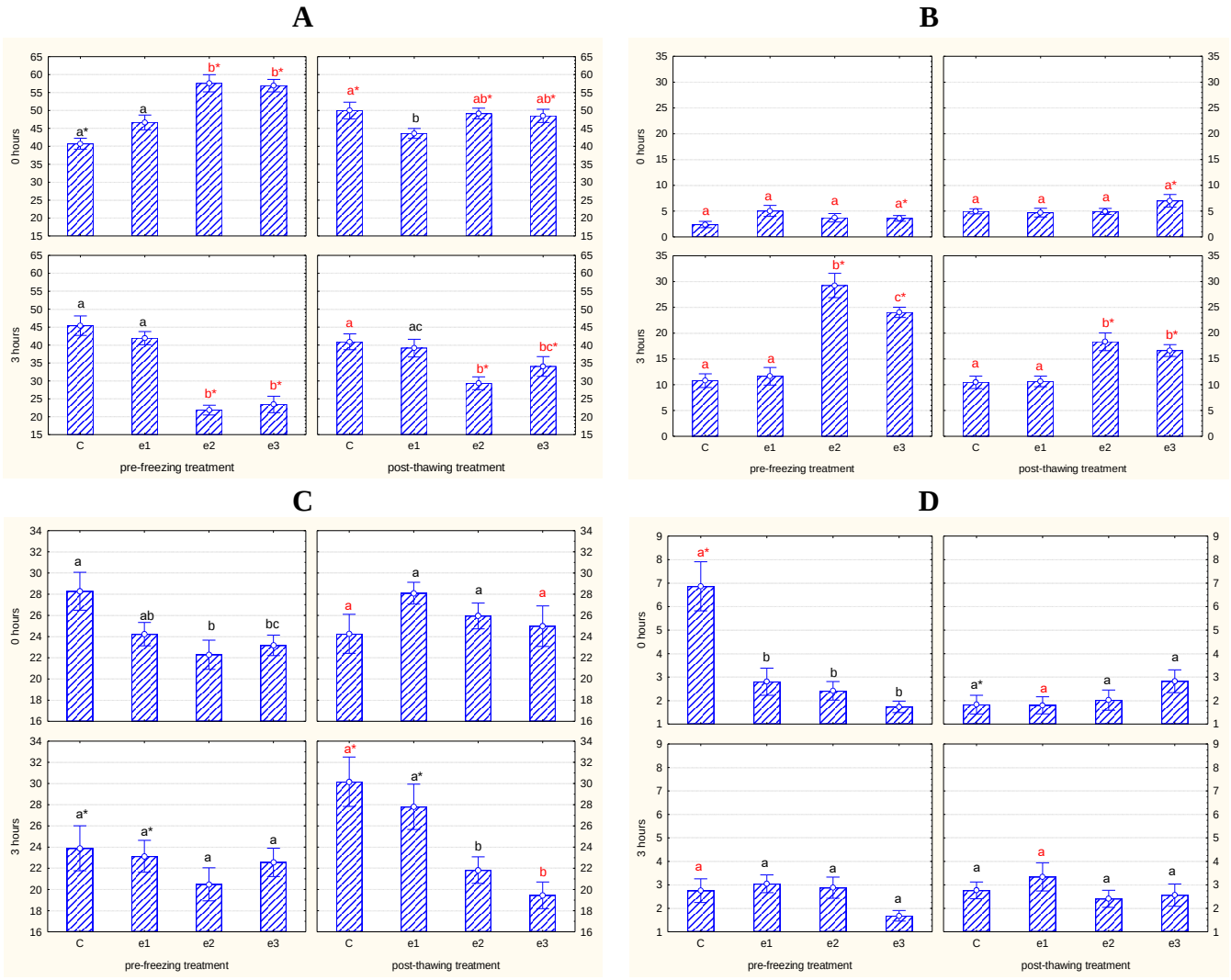

Figure 1. A. Viable, B. Damaged acrosome, C. Damaged plasma membrane, D. Pathological (mean % ±SE). Different letters (a-b) above the columns in the same sampling time and treatment, asterisk mark (*) between corresponding groups from different treatments and same sampling time, or red color (a-b) of letters between columns from different sampling time and same treatment represent significantly different values $p<0.05$

Motility

In the high-velocity cells assessment, E2-a-0 (29.75% ±3.06) had significantly lower value compared to the C-a-0 (45.73% ±3.94), E1-a-0 (43.60% ±2.20), and E3-a-0 (38.52% ±3.96). E1-a-0, E2-a-0, and E3-a-0 were significantly higher compared to their 3-hour counterparts E1-a-3 (31.21% ±3.14), E2-a-3 (17.67% ±1.92), and E3-a-3 (16.80% ±2.10). In the post-thawing treatment 0-hour samples, E3-b-0 (45.11%) was significantly higher compared to E2-b-0 (32.01% ±3.46). E3-b-0 was significantly higher compared to the E3-b-3 (34.51% ±2.70) samples. C-b-3 (36.02% ±3.96), E2-b-3 (33.75% ±3.40), and E3-b-3 were significantly higher

compared to C-a-3, E2-a-3, and E3-a-3, respectively. Additional information can be found in Figure 2-A.

In the motile cells pre-freezing treatment assessment, E2-a-0 (36.54% ±3.27) was significantly lower compared to C-a-0 (59.88% ±4.78), E1-a-0 (56.42% ±4.01), and E3-a-0 (50.49% ±4.27). The same was observed for E2-a-3 (23.91% ±2.09) compared to C-a-3 (50.25% ±5.63), E1-a-3 (46% ±3.27), and E3-a-3 (50.49% ±4.27). E2-a-0 was significantly higher than E2-a-3. In the post-thawing treatment, E2-b-0 (38.23% ±4.24) was significantly lower compared to C-b-0 (51.81% ±6.19) and E3-b-0 (50.76% ±3.13). E1-b-0 (41.30% ±4.41) was significantly lower compared to the E1-a-0. In the 3-hour samples, E3-b-3 (50.76% ±3.13) was significantly higher compared to C-b-3 (39.20% ±4.13), E1-b-3 (38.75% ±4.32), and E2-b-3 (37.51% ±3.78). E2-b-3 was significantly higher than its counterpart from the pre-freezing treatment E2-a-3. Additional information can be found in Figure 2-B.

In the progressively motile sperm assessment, the pre-freezing treatment samples showed that there was a significantly lower value in E2-a-0 (28.20% ±2.81) compared to C-a-0 (41.79% ±3.54), E1-a-0 (42.87% ±2.53), and E3-a-0 (36.27% ±3.64). In the 3-hour samples, E2-a-3 (16.95% ±1.87) was significantly lower compared to C-a-3 (41.43% ±4.53), E1-a-3 (28.15% ±2.64), and E3-a-3 (36.27% ±3.64). The E1-a-3 and E2-a-3 samples were significantly lower than their 0-hour counterparts E1-a-0 and E2-a-0, respectively. In the post-thawing treatment samples, E2-b-0 (29.66 ±3.09) was significantly lower compared to C-b-0 (41.79% ±5.28) and E3-b-0 (42.88 ±2.34). In the 3-hour samples, E3-b-3 (42.88% ±2.34) was significantly higher compared to C-b-3 (32.94% ±3.63), E1-b-3 (31.28% ±3.66), and E2-b-3 (32.08% ±3.10). E2-b-3 was significantly higher compared to its pre-freezing counterpart E3-a-3. Additional information can be found in Figure 2-C.

The pre-freezing treatment with SP has negatively affected the motility in terms of lower high-velocity, motile, and progressively motile sperm cells immediately following thawing. This was in line with the previous finding that the SP increases the capacitation dynamic and therefore lowers the motile sperm cells rendering the sample unfit for *in-vitro* or *in-vivo* use which coincides with the report of another study (de Graaf et al., 2007). Interestingly, the control did not affect the high-velocity, motile, and progressively motile sperm in the pre-freezing treatment following 3-hours of incubation. Compared to the experimental extenders, it could be suggested that the control would be most beneficial regarding these motility characteristics of the pre-freezing treatment samples, followed by the GSH/SP extender which negatively impacted high-velocity sperm cells. However, the treatment with GSH/SP in the post-thawing phase and following 3-hours of incubation was significantly beneficial to the motile and progressively motile sperm cells. This indicates that the E3 extender must be incubated with the samples following thawing to reach an observable effect and could be used to retain sperm motility for extended *in-vitro* or *in-vivo* insemination procedures concurring with previous studies (Barrios et al., 2000; El-Hajj Ghaoui et al., 2007; Pérez-Pé et al., 2001; Leahy & Gadella, 2011; Leahy & de Graaf, 2012). The GSH treatment in both pre-freezing and post-thawing samples did not cause any observable negative effect on motility traits of the sperm cells immediately following thawing, but this was quite the opposite in the 3-hour samples. It could be suggested that the effect of GSH on sperm motility is time-related and thus it should not be relied on for extended laboratory procedures partially concurring with the previous reports (Camara et al., 2011, Anel-Lopez et al., 2012).

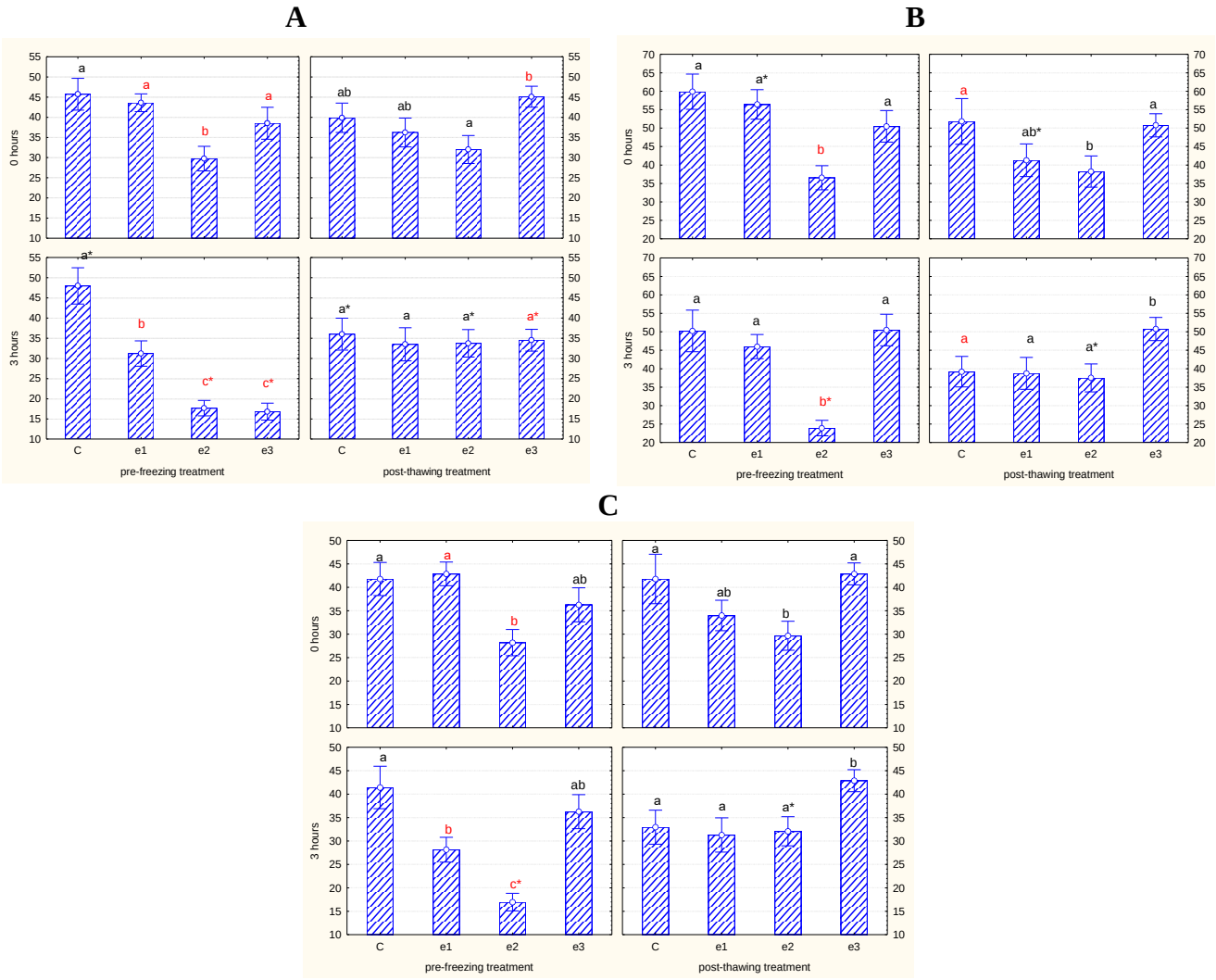

Figure 2. A. High-velocity, B. Motile, C. Progressively motile (mean % ±SE). Different letters (a-b) above the columns in the same sampling time and treatment, asterisk mark (*) between corresponding groups from different treatments and same sampling time, or red color (a-b) of letters between columns from different sampling time and same treatment represent significantly different values $p<0.05$

CONCLUSIONS

The pre-freezing treatment with SP and GSH/SP immediately following thawing yielded high sperm viability, lower incidence of plasma and acrosome membrane damage, and lower incidence of affected morphology compared to the control. This effect dissipated following 3-hours of incubation due to the increased acrosome damage. The pre-freezing treatment with SP has negatively affected the motility of thawed samples by lower high-velocity, motile, and progressively motile sperm cells. The control extender was the most beneficial for the motility characteristics of the pre-freezing treatment samples, followed by the GSH/SP extender which negatively impacted high-velocity sperm cells. The post-thawing treatment with GSH/SP

following 3-hours of incubation was significantly beneficial to the motile and progressively motile sperm cells. The effect of GSH on sperm motility was time-related and thus it should not be relied on for extended laboratory procedures. Ultimately, GSH/SP pre-freezing treatment immediately following thawing and GSH/SP post-freezing treatment with 3-hour incubation were most beneficial in yielding high viability and motility traits in cryopreserved ram sperm in liquid nitrogen.

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REFERENCES

Anel-López, L., Álvarez-Rodríguez, M., García-Álvarez, O., Álvarez, M., Maroto-Morales, A., Anel, L., ... & Martínez-Pastor, F. (2012). Reduced glutathione and Trolox (vitamin E) as extender supplements in cryopreservation of red deer epididymal spermatozoa. Animal reproduction science, 135(1-4), 37-46. Bailey, J. L., Bilodeau, J. F., & Cormier, N. A. T. H. A. L. Y. (2000). Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. Journal of andrology, 21(1), 1-7.

Barbas, J. P., & Mascarenhas, R. D. (2008). Cryopreservation of domestic animal sperm cells. Cell and Tissue Banking, 10(1), 49–62. doi:10.1007/s10561-008-9081-4

Barrios, B., Pérez-Pé, R., Gallego, M., Tato, A., Osada, J., Muiño-Blanco, T., & Cebrián-Pérez, J. A. (2000). Seminal Plasma Proteins Revert the Cold-Shock Damage on Ram Sperm Membrane1. Biology of Reproduction, 63(5), 1531–1537. doi:10.1095/biolreprod63.5.1531

Bucak, M.N., Ateşşahin, A., Varışlı, Ö., Yüce, A., Tekin, N., & Akçay, A. (2007). The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen. Theriogenology, 67(5), 1060–1067

Camara DR, Mello-Pinto MMC, Pinto LC, Brasil OO, Nunes JF, Guerra MMP (2011) Effects of reduced glutathione and catalase on the kinematics and membrane functionality of sperm during liquid storage of ram semen. Small Rumin Res 100:44–49

de Graaf SP, Evans G, Gillan L, Guerra MMP, Maxwell WMC & O'Brien JK 2007 The influence of antioxidant, cholesterol and seminal plasma on the in vitro quality of sorted and non-sorted ram spermatozoa. Theriogenology 67 217–227

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance OJ L 276, 20.10.2010, p. 33–79

El-Hajj Ghaoui, R., Thomson, P., Leahy, T., Evans, G., & Maxwell, W. (2007). Autologous whole ram seminal plasma and its vesicle-free fraction improve motility characteristics and membrane status but not in vivo fertility of frozen-thawed ram spermatozoa. Reproduction in Domestic Animals, 42(5), 541–549.

García-Álvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Garde, J. J., Ramón, M., Fernández-Santos, M. R., Soler, A. J. (2009). Sperm characteristics and in vitro fertilization

ability of thawed spermatozoa from Black Manchega ram: Electroejaculation and postmortem collection. Theriogenology, 72(2), 160–168
Leahy, T., & Gadella, B. M. (2011). Sperm surface changes and physiological consequences induced by sperm handling and storage. Reproduction, 142(6), 759.
Leahy T & de Graaf SP 2012 Seminal plasma and its effect on ruminant spermatozoa during processing. Reproduction in Domestic Animals 47, 207–213.
Leahy, T., Rickard, J. P., Bernecic, N. C., Druart, X., & de Graaf, S. P. (2019). Ram seminal plasma and its functional proteomic assessment. Reproduction, 157(6), R243-R256.
Marti, E., Mara, L., Marti, J. I., Muiño-Blanco, T., & Cebrián-Pérez, J. A. (2007). Seasonal variations in antioxidant enzyme activity in ram seminal plasma. Theriogenology, 67(9), 1446-1454.
Morrier, A., Castonguay, F., & Bailey, J. L. (2003). Conservation of fresh ram spermatozoa at 5 C in the presence of seminal plasma. Canadian journal of animal science, 83(2), 221-227.
Muiño-Blanco, T., Pérez-Pé, R., & Cebrián-Pérez, J. A. (2008). Seminal plasma proteins and sperm resistance to stress. Reproduction in domestic animals, 43, 18-31.
Nikolovski, M., Dovenska, M., Ilievska, K., Adamov, N., Atanasov, B., Radeski, M., Kirovski, D., Petkov, V., & Dovenski, T. (2019). Homologous seminal plasma and glutathione promote pre-capacitation motility and structural stability of cryopreserved ram spermatozoa. Mac Vet Rev 42(2), 169-179
Pérez-Pé, R., Cebrián-Pérez, J. A., & Muiño-Blanco, T. (2001). Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa. Theriogenology, 56(3), 425-434.
Pini, T., Leahy, T., & de Graaf, S. P. (2018). Sublethal sperm freezing damage: Manifestations and solutions. Theriogenology, 118, 172–181. doi:10.1016/j.theriogenology.2018
Silva S, Soares A, Batista A, Almeida F, Nunes J, Peixoto C, Guerra M (2011) In vitro and in vivo evaluation of ram sperm frozen in tris egg-yolk and supplemented with superoxide dismutase and reduced glutathione. Reprod Domest Anim 46:874–881
Tseng, H. C., Lee, R. K. K., Hwu, Y. M., Lu, C. H., Lin, M. H., & Li, S. H. (2013). Mechanisms underlying the inhibition of murine sperm capacitation by the seminal protein, SPINKL. Journal of Cellular Biochemistry, 114(4), 888-898.
World Health Organization (2010). WHO Laboratory Manual for the Examination of Human Semen. In: Cooper, T.G. (Ed.). Standard procedures (pp. 37). WHO Press
Zeitoun, M. M., & Al-Damegh, M. A. (2014). Effect of nonenzymatic antioxidants on sperm motility and survival relative to free radicals and antioxidant enzymes of chilled-stored ram semen. Open Journal of Animal Sciences, 5(01), 50.