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Cooling storage of ram sperm in presence of antioxidant glutathione

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Abstract: In the process of cryopreservation, the semen of farm animals can be threatened by many factors, such as oxidative damage, which affect the motility and plasma membrane function of the sperm. As an endogenous antioxidant in animals, glutathione (GSH) can significantly improve the quality of thawed sperm when added to the diluent of the semen. This study was aimed at examining the effect of GSH (given at 0.5 mM, 1.5 mM and 5 mM) on the motility and viability of ram sperm following storage under cooling conditions for several days. GSH reduced the negative effect of the cooling conditions on the total and progressive motility (CASA), stability of the membranes (annexin V/PI) and incidence of the apoptosis/necrosis (Yo-Pro-1/PI) in the sperm maintained at 5 °C for 24 h and 48 hours. However, this effect was neither reflected in the penetrating nor in the fertilising ability (zygotic cleavage rate) of the sperm examined in a heterologous (bovine oocytes and ram sperm) *in vitro* fertilisation test with GSH applied at 1.5 mM for 48 h of cooling storage. These results indicate that the addition of GSH to the sperm extender can maintain the quality of the ram sperm stored at cooling conditions for at least two days. To make a definite conclusion about the GSH effect on the sperm functionality (fertilising ability), other GSH concentrations should be tested.

Keywords: sheep; spermatozoa; motility; viability; membrane; apoptosis

The long-term storage of ram sperm is provided by deep freezing in liquid nitrogen using either a programmable regime or a manually operated one. Such a procedure requires special equipment, but the post-thaw survival of ram sperm is relatively low because of the high sensitivity of ram sperm membranes to freezing and thawing. However, very often, ram semen doses should be kept only a couple of days prior to use for ewe insemination. In such cases, ram sperm can be stored in a liquid form (in an appropriate extender) at a temperature not higher than 5 °C for several days. Generally, cooled semen has greater longevity in the female tract than frozen semen (Sieme et al. 2003), although it may be species-specific. In particular, very good pregnancy rates were obtained using frozen stallion semen for artificial insemination (AI) in the right phase of a mare's oestrous cycle, which was comparable with those using chilled semen (Gaspardy et al. 2020). Therefore, the prop-

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er approach to sperm storage rather depends on the animal species used.

The most common problem during the semen chilling process is the damage to the sperm membranes as a result of lipid peroxidation and the excessive formation of unsaturated fatty acids. Mammalian spermatozoa are rich in unsaturated fatty acids and easily subjected to reactive oxygen species (ROS) formation that can reduce the spermatozoa motility and impair the acrosome reaction and sperm capacitation (Sikka 1996). To prevent ROS formation and to avoid oxidising processes during liquid storage, different antioxidant substances may be used.

The addition of antioxidants is well known to improve the viability and motility of post-thawed ram sperm (Baumber et al. 2005). One of those such substances is glutathione (GSH), which is known to act also as a cryoprotectant during semen freezing (Munsi et al. 2007) and to assist in antioxidant defence mechanisms during semen thawing (Gadea et al. 2004). Numerous studies about GSH additions into semen extenders have reported controversial results (Silvestre et al. 2021). Particularly in rams, the majority of the studies found no effects of extender supplementation with GSH (see Silvestre et al. 2021). It is possible that the crucial factor in the case of GSH is its concentration, which may be variable in different animal species. For example, in bulls and boars, the optimal GSH dose was reported to be in the range between 0.5 mM and 1.5 mM (Foote et al. 2002; Munsi et al. 2007; Zhang et al. 2016), whereas in rams these optimal doses were much higher than in other species (Shi et al. 2020).

The goal of this study was to examine the effect of glutathione (GSH), given at three different doses, on the motility and selected viability parameters (membrane integrity, membrane stability, apoptosis) of ram sperm following liquid storage under cooling conditions.

MATERIAL AND METHODS

Experimental design

All the experiments were carried out with fresh ram spermatozoa, collected from four Lacaune breed sexually mature rams at 2.5–3.0 years of age using an artificial vagina within the autumn season (September–November). These rams were involved into the breeding process, and frequency of the sperm collection was once or twice per week. The rams were kept under uniform nutritional conditions. The quality of the ram sperm was assessed shortly after collection. Only the ejaculates, which fulfilled the following criteria, were accepted: volume – 0.5 cm^3, sperm concentration > 2.8 × 10^6/mm^3, motility > 70%, morphological abnormalities < 20%.

After measuring the volume, density and activity, the sperm was diluted (approximately 1 : 3) in a Triladyl extender (Minitub Slovakia Ltd, Čeľadice, Slovak Republic) containing 20% egg yolk, 10% lactose and 6% glycerol to obtain a concentration of 1 × 10^6 sperm/ml. The ejaculates from three of the best rams were selected according to their quality per each experiment and were cooled to 5–7 °C and transported to the laboratory in a thermo box within two hours. In the laboratory, the ejaculates were flushed out of the Triladyl extender using centrifugation at 600 g at the laboratory temperature (20–23 °C) for 7 min and resuspended in a Tris extender (Minitub Slovakia Ltd, Čeľadice, Slovak Republic) without any egg yolk. In order to avoid any individual differences among the rams, the obtained sperm samples from each ram were pooled together to make heterospermia and then divided into four groups with 1 ml of the ejaculate in each group and adjusted to a final concentration of 1 × 10^6/ml. Afterwards, glutathione (GSH; Sigma-Aldrich, St. Louis, MO, USA) was added to the marked tubes at concentrations of 0.5 mM; 1.5 mM or 5 mM; the control group did not contain glutathione (0 mM). The samples were stored in polystyrene tubes at cooling conditions (4 °C in a fridge) for 48 hours.

Analyses of the motility parameters were performed at 1 h, 24 h and 48 h of sperm cooling-storage. Following this storage period, the sperm samples were removed from the refrigerator, washed to remove the extender using centrifugation (400 *g*), resuspended in a saline solution (0.9% NaCl) that contained 1% of a foetal calf serum (saline-FCS) and analysed. The sperm motility was analysed at the time intervals of 0 h, 0.5 h or 2 hours. Between these time points the sample were incubated at 37 °C. The sperm penetration/fertilisation ability was examined in an *in vitro* fertilisation (IVF) test after 48 h of cooling storage in the presence of GSH at 1.5 mM.

The viability parameters (membrane integrity, membrane stability, apoptotic/necrotic sperm) were analysed following 48 h of sperm storage.

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Analysis of the sperm motility

The total sperm motility was measured on the 1st (1 h), 2nd (24 h) and 3rd (48 h) days following the GSH addition using a computer-assisted sperm analysis (CASA) system (Sperm Vision®, Minitub, Slovakia Ltd, Čeľadice, Slovak Republic) at 0 h, 0.5 h and 2 h after removal from storage. The sperm samples were diluted in a saline solution containing 1% of the foetal calf serum (saline-FCS) and transferred by pipette into a Leja counting chamber with a depth of 10 µm. The chamber was placed under a Zeiss Axioscope A.1 phase-contrast microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) with a heating plate (37 °C) at 200× magnification. In each sample, at least six view fields were counted. Of all the parameters measured by the CASA, only the total motility and progressive movement of the sperm were evaluated in this study. The values of the total or progressive motility measured at several time points (0 h, 0.5 h and 2 h) during the day were summarised and the average values per each day were represented in the graphs.

Viability assays

Following 48 h of cold storage, the sperm samples were analysed for the plasma membrane integrity (PNA-FITC), membrane stability (annexin V-Fluos), sperm death (propidium iodide; PI) and apoptosis (Yo-Pro-1) using fluorescent assays.

The sperm acrosome membrane integrity was evaluated by fluorescently labelled lectin PNA-Alexa Fluor (peanut agglutinin; Molecular Probes, Lucerne, Switzerland) in combination with propidium iodide, which detected the dead or necrotic sperm in the samples (red signal) and DAPI – blue fluorescent DNA stain (H-1200; Vector Laboratories Inc., Burlingame, CA, USA), which marked all the sperm cells in the samples. The sperm samples were incubated in a staining solution that contained 20 µmol/l of PNA-Alexa Fluor and 5 µg/ml of PI in a saline-FCS solution for 20 minutes. Following this time, the samples were examined under a Leica fluorescent microscope (MIKRO s.r.o., Bratislava, Slovak Republic) with filters for green, red and blue fluorescence. Only the sperm heads with a damaged (acrosome-reacted) membrane were marked by green fluorescence, whilst the sperm with intact membranes remained unstained.

The apoptotic sperm were detected using specific nuclear green fluorochrome, Yo-Pro-1 (Molecular Probes, Lucerne, Switzerland) in combination with PI. Following washing in the saline-FCS solution, the sperm samples were incubated for 20 min at room temperature in the staining solution containing 5 µmol/l of Yo-Pro-1 and 5 µg/ml of PI in 200 µl of the saline-FCS solution. The sperm cells with a bright green fluorescent signal were regarded as apoptotic.

The sperm membrane phosphatidylserine (PS) translocation (membrane stability) was detected using fluorescently labelled Annexin V-Fluos (Roche Slovakia Ltd, Bratislava, Slovak Republic). The sperm samples were prepared as described before by Makarevich et al. (2011). Sperm with PS translocation exhibited green fluorescence and was regarded as sperm with membrane destabilisation. The presence of annexin V-positivity in the sperm was localised on the acrosomal part of sperm head, the post-acrosomal segment, an equatorial segment and the sperm membrane along the whole head (Makarevich et al. 2011).

The proportion of dead spermatozoa to the total sperm population was determined after staining with propidium iodide (PI; 5 µg/ml) and DAPI fluorescent dye, a component of the Vectashield antifade reagent (H-1200; Vector Laboratories, Inc., Newark, CA, USA), which labels the chromatin of all the sperm cells.

Analysis of the sperm penetrating/fertilising ability

The sperm penetration/fertilisation ability was determined after 48 h of cold storage with or without (control group) the GSH (1.5 mM) by an *in vitro* fertilisation (IVF) test on bovine pre-matured oocytes isolated from the ovaries of cows that had been provided by a local slaughterhouse. The ability of bovine oocytes with *zona pellucida* to be fertilised by ram sperm has been validated previously (Garcia-Alvarez et al. 2009). Briefly, isolated oocytes were *in vitro* matured (IVM) during 24 h incubation as described previously (Chrenek et al. 2017). Following the IVM procedure, the oocytes were stripped of the cumulus cells using vortexing and placed under mineral oil into a fertilisation drop (Fert-TALP medium with 10 µg/ml of heparin). The sperm suspension was washed in a Sperm-

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TALP medium to remove the Triladyl, resuspended in a fresh Fert-TALP medium that contained the above-mentioned additions and placed into a fertilisation droplet to a final concentration of 2 × 10^6 sperm/ml. Fertilisation was performed in the incubator at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 20 hours. Subsequently, the presumptive zygotes were cleaned of the excessive sperm and the rest of the cumulus cells by vortexing and transferred into a B2 INRA culture medium (CCD Laboratories, Vernouillet, France) on a culture dish with a monolayer of buffalo rat liver cells. The zygotes were cultured at 38.5 °C in a humidified atmosphere with 5% CO_2 in air until the evaluation.

After four days, the zygotes and embryos were fixed in 3.7% w/v formalin, mixed in a droplet of Vectashield with DAPI, mounted between a microslide and a coverslip and inspected under a Leica fluorescence microscope with the respective UV filter. The zygotes and embryos were evaluated as follows: (I) penetrated eggs – at least one sperm under the *zona pellucida* and one pronucleus were present; (II) fertilised eggs – at least two pronuclei were present; (III) cleaved embryos – two or more blastomeres with visible nuclei were present; and (IV) non-penetrated oocytes – with no spermatozoa inside. The penetrating ability was calculated as the sum of oocytes with at least one sperm inside the ooplasm (1 Sp), one sperm + one or two pronuclei (1 PN/Sp + 2 PN), polyspermic oocytes (> 2 PN) and cleavaged zygotes to the total number of oocytes. The fertilising ability was calculated as the sum of 1 PN/Sp, 2 PN, polyspermic oocytes and cleavaged zygotes to the total number of oocytes.

Statistical processing

The experiments were performed in four replications. For the sperm motility analysis, seven view fields in each experimental group were evaluated, so that at least 500–1 000 sperm cells per one experimental group were counted. The average values were calculated from three measurements (0 h, 0.5 h and 2 h) during the day. Comparisons of the arithmetic means between groups of GSH concentrations were performed by a one-way analysis of variance (ANOVA) with fixed effects: the first effect was the exposure time; the second effect was the GSH concentration. The results of total or progressive motility, obtained from several measurements, were statistically processed by a repeated-measure ANOVA. For the viability fluorescent assays, at least eight view fields in each experimental group were evaluated (more than 800 sperm cells). Comparisons of the arithmetic means between groups of GSH concentrations were performed by a one-way ANOVA. The elementary contrast between the mean values of the fluorescent assays was evaluated by the Bonferroni test. The penetration/fertilisation test was performed in four replications, and the obtained data were analysed using the Chi-square test. Since the percentage values were used in the study, all the values were subjected to the arcsin transformation in order to eliminate any abnormality of the distribution and/or non-homogeneity of the data. The statistical analysis was performed with the original data using the Statistix analytical software (v8.0; https://www.statistix.com/)

RESULTS

Effect of the GSH on the sperm motility

The average values of the ram sperm total motility and progressive movement following 1 h, 24 h or 48 h of cooling storage were evaluated. As can be seen from Figure 1, the short-term incubation (1 h) of the ram sperm with glutathione (GSH) had no effect on both the total and progressive motility. However, at longer exposures (24 h and 48 h), the GSH influenced both the total and progressive motility. Higher concentrations (1.5 mM and 5 mM) had a considerable effect on the total sperm motility after 24 h of storage. However, a more expressed GSH effect was observed after 48 h of sperm incubation, when all the tested GSH doses significantly increased both the total motility and progressive movement. A significant increase in the total motility was noted at the highest GSH concentration (5 mM), when the total motility increased from 70.59% (control group) to 87.20% ($P < 0.05$). Similar tendencies were observed for the progressive movement; the GSH given at 5 mM significantly improved the proportion of progressively moving sperm from 65.36% (control group) to 82.93% ($P < 0.05$). Furthermore, the positive effect of the GSH was visible also at a moderate dose (1.5 mM), where the propor-

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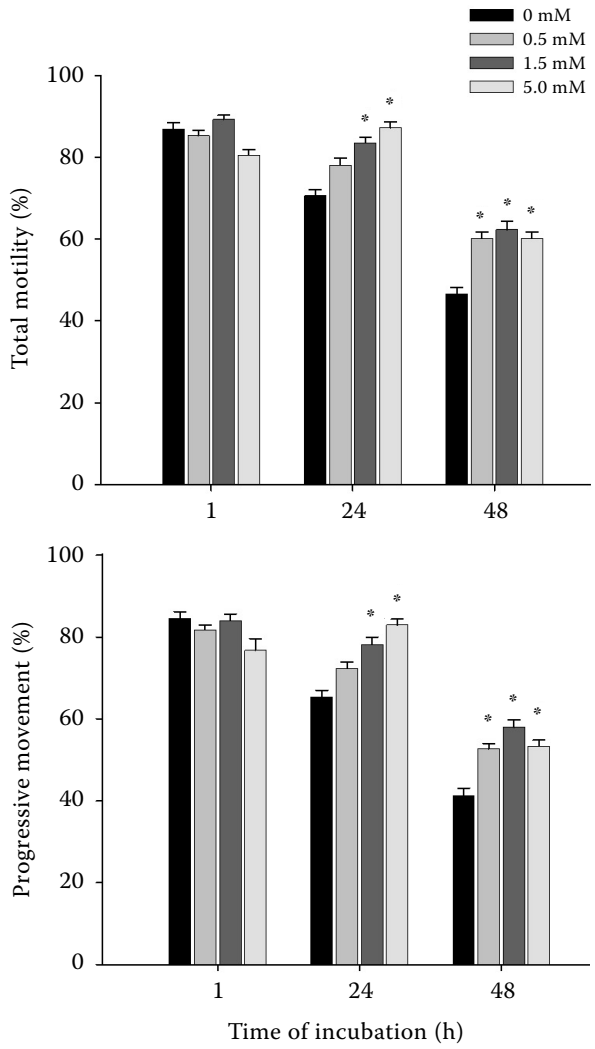

Figure 1. Effect of glutathione (GSH) on the ram sperm motility

*Significant differences compared to the control (0 mM) at $P < 0.05$

tions of the motile or progressively moving sperm were 83.52% and 78.19%, respectively. The GSH given at the lowest dose (0.5 mM) neither promoted the total motility nor the progressive movement of the ram sperm, demonstrating its dose-dependent action.

Effect of the GSH on the sperm viability

The integrity of the sperm acrosomal membranes, labelled with PNA-FITC (in a range of 22.65–24.75%), was not significantly affected by any of the GSH doses (Table 1). The occurrence of necrotic sperm with a disrupted acrosomal membrane (PNA+/PI+; 7.8% in the control) was significantly suppressed by 0.5 mM of GSH, whilst higher doses did not show any effect.

About 15% of the sperm cells in the control group were apoptotic (Yo-Pro-1+). GSH suppressed the occurrence of apoptotic spermatozoa, when given at moderate (1.5 mM) or higher (5.0 mM) doses. The percentage of both apoptotic/necrotic spermatozoa (Yo-Pro-1+/PI+) in the control group (10.5%) was significantly decreased by the moderate (1.5 mM) or higher (5.0 mM) GSH doses (Table 1).

About 13% of the spermatozoa showed membrane destabilisation, revealed by the disruption of an annexin V-labelled phosphatidylserine (PS) asymmetry. GSH lowered this destabilisation when given at all the tested doses. The percentage of necrotic sperm with disrupted PS asymmetry (about 11%) was not significantly affected by any of the GSH doses (Table 1).

Table 1. Effect of the glutathione (GSH) on the sperm viability (%, mean ± SEM)

Viability marker	Glutathione (mM)			
	0 mM	0.5 mM	1.5 mM	5.0 mM
Acrosomal membrane integrity				
PNA-FITC+	22.65 ± 2.12	22.76 ± 1.93	24.75 ± 2.63	24.04 ± 0.58
PNA-FITC+/PI+	7.80 ± 0.97	4.08 ± 1.02*	6.40 ± 1.00	7.29 ± 0.70
Apoptosis				
Yo-Pro-1+	15.10 ± 1.64	12.5 ± 0.70	8.78 ± 2.08*	7.59 ± 1.38*
Yo-Pro-1+/PI+	10.51 ± 1.31	10.43 ± 1.18	6.08 ± 1.04*	6.25 ± 1.06*
Membrane stability				
Annexin V+	12.82 ± 0.89	7.42 ± 0.64*	5.63 ± 1.47*	7.84 ± 1.17*
Annexin V+/PI+	10.92 ± 1.36	8.08 ± 1.03	8.61 ± 0.77	9.00 ± 1.46

*Significant differences compared to the control (0 mM) at $P < 0.05$

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Effect of the GSH on the sperm penetration and fertilising ability using an IVF test on bovine oocytes

The penetrating ability in the control sperm (without GSH) was about 94% and this parameter was not significantly affected by the GSH addition (Table 2). The cleavage of bovine zygotes was not significantly influenced by the GSH addition, but was only slightly higher than in the control (no GSH). Similarly, the fertilising ability was only slightly higher after the GSH addition compared to the control and this effect was not significant.

DISCUSSION

Antioxidants, particularly glutathione, play an essential role in sperm protection against oxidative stress (Abdullah et al. 2021), which may improve the sperm viability and fertilising ability (Bucak and Tekin 2007; Wu et al. 2021). Although numerous reports about the use of GSH as an antioxidant in semen extenders are known, the results still remain controversial because of the very wide range of GSH concentrations used in various studies. The majority of the studies reported either no effects or detrimental effects of the extender supplementation with GSH at high concentrations (50–400 mM), while most of the positive effects on ram and bull sperm were reported using middle (5–10 mM) or low (0.2–5 mM) GSH concentrations (Silvestre et al. 2021). Probably, the reported effects of GSH in various studies were greatly influenced by the concentrations as well as the examined animal species. In particular, GSH promoted motility and viability of ram sperm at 100 mM and 200 mM (Camara et al. 2011; Shi et al. 2020), but caused negative effects at 400 mM (Camara et al. 2011). The GSH also improved mitochondrial activity (Mata-Campuzano et al. 2014) and survival of ram sperm (Bucak and Tekin 2007), retained optimal motility of bull (Munsi et al. 2007) and rabbit (Ahmad et al. 2021) sperm at moderate doses (2–10 mM), whereas given at lower doses (0.5–1.5 mM), it had no effect on bull (Foote et al. 2002) and tigrina (Angrimani et al. 2017) sperm.

The results of the antioxidant supplementation also varied according to the storage length. Beneficial effects were normally observed during long storage periods, while no clear effects were observed during the first hours of liquid (cooling) storage (Foote et al. 2002; Perumal et al. 2013; Perumal 2014; Del Prete et al. 2019; Jofre et al. 2019; Silvestre et al. 2021). Nevertheless, Bucak and Tekin (2007) reported the positive effect of GSH (5–10 mM) on ram sperm survival during 6 h of storage at 5 °C.

In our study, we examined the effect of the GSH addition to sperm after 24–48 h (sperm motility), 48 h (penetration/fertilisation ability) or 72 h (viability assays) of cooling storage at 4 °C in a Triladyl extender. Our results indicate the highest effect of the GSH after 24 h of cooling storage, when GSH, given at 1.5 mM and 5 mM, improved the total sperm motility and progressive movement. After 48 h of cooling storage, a positive effect of the GSH on the total and progressive motility was recorded at all the tested concentrations. The data indicate that glutathione can maintain good quality of movement of ram sperm during long-term cooling storage. It is generally accepted that the motility decreases with length of storage. As can be seen from our data, a GSH concentration of 1.5 mM is already sufficient to maintain sperm cooled to 5 °C to be motile up to 48 hours.

Besides the length of storage, the influence of the GSH concentration on the sperm survival is obviously species-specific. In particular, GSH, added at 0.5 mM to bull sperm diluents, was effective to protect the plasma membranes and maintain the motility of chilled sperm at 5 °C (Triwulanningsih et al. 2008). Munsi et al. (2007) maintained the op-

Table 2. Effect of the glutathione (GSH) (1.5 mM) on the penetrating/fertilising ability of the ram sperm, *n* (%)

Group of sperm	Non-penetrated oocytes	Sperm inside the oocyte	1 PN/Sp + 2 PN	Polyspermic > 2 PN	Zygote cleavage	Penetrating ability	Fertilising ability
Control (*n* = 68)	4 (5.88)	12 (17.65)	16 (23.53)	2 (2.94)	34 (50.0)	64 (94.12)	52 (76.47)
GSH (*n* = 84)	7 (8.33)	10 (11.90)	20 (23.81)	1 (1.19)	46 (54.76)	77 (91.66)	68 (80.95)

Penetrating ability = at least one sperm inside the oocyte + (1 PN/Sp + 2 PN) + polyspermic (> 2 PN) + cleavaged zygotes; fertilising ability = 1 PN/Sp + 2 PN + polyspermic + cleavaged zygotes

Differences between the control and the GSH groups are not statistically significant ($P > 0.05$; Chi-square test)

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timum bull sperm motility (around 50%) for three days by GSH additions in a range of 0.5–2.0 mM. The quality of buffalo sperm was improved during 120 h of liquid storage by adding 0.5 mM or 1.0 mM of GSH to a semen diluent (El-kon and Darwish 2011). Oppositely, no effects of GSH at 5 mM on the progressive movement of post-thawed boar sperm (Whitaker et al. 2008) and on human sperm PM and other CASA movement parameters (VSL, VAP, VCL, ALH) over a 4 h cooling storage period (Donnelly et al. 2000) were reported. Similarly, no significant effect on the motion parameters (VCL, VSL, VAP, ALH) of boar cryopreserved sperm was found after the addition of GSH to the thawing medium (Gadea et al. 2004). However, our present results on ram sperm indicate that higher concentrations of GSH (1.5 mM and 5 mM) are more beneficial for ram sperm movement than lower concentrations (0.5 mM).

Sperm viability is greatly dependent on the status of the sperm membranes and directly affects the fertilising ability of the sperm (Zou et al. 2021). Glutathione improved the functionality of the plasma membrane in the sperm of mice (Abdullah et al. 2021), chilled sperm of dogs (Andersen et al. 2018) and cryopreserved sperm of goats (Zou et al. 2021). In cryopreserved sperm, the integrity of the ram plasma membrane was not affected by higher GSH doses (Bucak et al. 2008), whilst low concentration of GSH (2 mM) improved the acrosomal integrity of goat sperm (Zou et al. 2021). According to Ahmad et al. (2021), moderate GSH doses (2–4 mM) are beneficial to rabbit sperm chill stored for several hours in an extender, due to the weak antioxidant system in the rabbit sperm, whilst a higher dose of GSH (8 mM) caused an inadequate response for the sperm quality.

In our study on chilled ram sperm, we observed that GSH supported the sperm viability during a three-day cooling storage period. Thus, given either at lower, moderate or higher doses, GSH suppressed the incidence of apoptotic/necrotic sperm (Yo-Pro/PI), membrane destabilised sperm (annexin V) and necrotic sperm with a destabilised plasma membrane (annexin V/PI). A concentration of 1.5 mM GSH showed a protective effect on the sperm plasma membrane stability, as 94.37% of the sperm retained phosphatidylserine (PS) asymmetry (5.63% of sperm were annexin V-positive) compared to 87.18% PS asymmetry in the control group (12.82% of sperm were annexin V-positive). The percentage of acrosome-reacted sperm (with disrupted membranes; PNA+/PI+) was not affected by any GSH concentration.

When assessing the sperm quality, in certain cases, the motility may not reflect the functional status. Therefore, it is recommended to evaluate the fertilisation capacity of the spermatozoa by an *in vitro* penetration test on animal eggs. It is generally known that mammalian oocytes are protected against the entry of foreign sperm by a species-specific barrier represented by the *zona pellucida*. Slavik et al. (1990) showed that ram sperm are able to penetrate bovine oocytes with the subsequent formation of pronuclei. Moreover, they proved that the *zona pellucida* of bovine and sheep oocytes does not represent a barrier against the penetration of goat sperm. In our study, we used a heterologous species model to examine the penetration and fertilisation (embryo cleavage) ability of ram sperm in an IVF test on bovine slaughterhouse-derived oocytes. The GSH in our experiments, given at 1.5 mM, neither changed the penetrating ability nor the cleavage rate of zygotes and sperm fertilising ability compared to the control without GSH. However, we cannot make an unambiguous conclusion about the inefficiency of GSH on the sperm fertilising ability using only one concentration, therefore, further trials are required using other GSH concentrations and (perhaps) a homologous species model (ram sperm-sheep oocytes).

CONCLUSION

In our study, GSH reduced the negative effect of the cooling conditions on the total and progressive motility, stability of the membranes and incidence of apoptosis/necrosis in the sperm maintained at 5 °C for 24 h and 48 hours. These results indicate that addition of GSH to the sperm extender can maintain the quality of the ram sperm stored at cooling conditions for at least two days.

Conflict of interest

The authors declare no conflict of interest.

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