Seminal Plasma Proteins Prevent Detrimental Effects of Ram Sperm Cryopreservation and Enhance the Protective Effect of Lecithin

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Abstract

Background: One of the main drawbacks of refrigerated and frozen-thawed semen is lipid peroxidation and the subsequent generation of reactive oxygen species from cellular metabolism. In this study, we tested the hypothesis that the use of antioxidant compounds improves the frozen-thawed sperm quality. We analysed the effect of different antioxidants, or antioxidant-like additives, upon the processes involved in cooling and freeze-thawing ram semen.

Methods: Sperm motility, plasma membrane integrity and stability, and mitochondrial membrane potential (MMP) were tested immediately after thawing and following incubation for 3 h and 6 h at 37°C.

Results: The addition of oleic/linoleic acid did not improve sperm viability, although it resulted in increased motility after refrigeration and rewarming, similar to that found with pyruvic acid. In frozen-thawed samples, the effect of 75 mM ascorbic acid was beneficial and improved viability, membrane stability, MMP and motility. Pyruvic acid, melatonin, pinoline, and N-acetyl cysteine, or the combination of certain antioxidants such as oleic/linoleic acids with tocopherol, lipoic and ascorbic acids, melatonin and pinoline, N-acetyl cysteine and GSH did not significantly improve the frozen-thawed sperm quality. Thawed samples supplemented with lecithin scored higher (p<0.001) membrane integrity and stability and MMP values than controls. However, the alterations induced in the inner mitochondrial membrane resulted in a very low proportion of functional mitochondria in a time-dependent manner after thawing. These detrimental effects were prevented by seminal plasma proteins, which enhanced the protective effect of lecithin.

Conclusion: Seminal plasma proteins strengthened the cryoprotective ability of lecithin and not only were sperm viability and membrane stability well maintained, but mitochondrial functionality was preserved.

General significance: Lecithin together with seminal plasma proteins may be used as cryoprotectants for ram semen.

Keywords: Ram spermatozoa; Antioxidants; Mitochondrial potential; Cryopreservation

Introduction

Sperm cryopreservation is an important component of the success of assisted reproductive technologies. Despite the progress on fundamental cryobiology, the methodology applied to develop sperm cryopreservation protocols remains mostly empirical, because no correlation between data derived from theoretical work and the observed results has been established [1,2]. The improvement of semen cryopreservation protocols implies the need to study, along with classic sperm quality parameters, alternative markers that provide a better understanding of cell cryoinjury. Furthermore, in order to minimize the risk of animal disease transmission, alternatives to egg yolk for the dilution media must be explored. Commercial extenders with egg yolk substitutes have been assayed for freeze-thawing ram semen. Bioexcell\textsuperscript{1}, a diluent that contains no additives of animal origin, was shown to preserve sperm quality parameters [3] to a level comparable to the conventional milk-egg yolk extender [4,5], and provided similar fertility results after cervical AI, although individual variations were significant [6].

The cryopreservation process is an unnatural and stressful methodology that provokes irremediable cryoinjuries to spermatozoa, which structure and physiology is not designed to resist. These cryostresses start with temperature decreases and follow with induced lipid peroxidation and the loss of antioxidant enzyme activity. Therefore, antioxidants have been considered as important components of semen diluents, even though the results of several studies are contradictory and have shown, a lack of effect in some cases [7-10], while an improvement in post-thawing sperm quality has been found in other cases [11-19]. This could be due to the high heterogeneity of antioxidant molecules (lipids, proteins, enzymes, organic acids among others) and the subsequent wide variety of their action mechanism [19-31]. Basically, antioxidants are needed to maintain adequate, but not excessive, levels of reactive oxygen species (ROS) [32].

In this study, we investigated the hypothesis that the use of antioxidant compounds may increase the frozen-thawed sperm quality. Therefore, the objective was to evaluate the effect of possible egg yolk substitutes in the extender during cooling or freeze-thawing of ram semen. We have analysed the effect of different antioxidants, or antioxidant-like compounds, in both cooling and freezing ram semen processes. The compounds used were chosen on the basis of their protective effects. Ascorbic acid is a ROS scavenger and membrane protector associated with vitamin E [33-35]; α-lipoic acid has an inhibitory effect on Pyruvate dehydrogenase complex and functions as

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a vitamin C regenerator in the presence of glutathione [36,37]; N-acetyl cysteine is a ROS scavenger, SH groups donor and GSH precursor [38]; reduced glutathione (GSH) is another ROS scavenger that removes lipidic and hydrogenated peroxides and prevents oxidation of biomolecules. Furthermore, GSH regulates the intracellular redox status and mitochondrial functionality [39]. Melatonin is an antioxidant molecule [40,41] that modulates sperm functionality [42] and capacitation [43] and reduces mitochondrial membrane potential inhibiting the production of the anion superoxide and hydrogen peroxide; likewise, it inhibits the nitric oxide synthase [44] and can inhibit apoptosis [45]; pinolone reduces lipid peroxidation due to oxidative stress like melatonin [46]; α-tocopherol is a ROS scavenger associated to lipidic membrane with preventive action crucial [49]. Trehalose provides energy substrate for the sperm cells while also acting as a cryoprotectant and rendering the spermatozoa capable of tolerating freeze-thawing damage [50-52]. In this study, we have determined the effect of two different pyruvate concentrations and, at the same time, whether trehalose could be a more efficient protective agent than sucrose. The use of soy lecithin (L-a-phosphatidylcholine) has been demonstrated to be safer than egg yolk in terms of biosecurity [53] and to have neither a cytotoxic effect [54] nor a negative effect on sperm motility [55]. Lecithin is a normal constituent of the sperm plasma membrane [56] and the use of lecithin-containing extenders has been useful for maintaining high quality values in refrigerated [57] and frozen-thawed [6] ram spermatozoa [13,14, 17-19, 57-61]. Likewise, lecithin-based semen extenders have been associated with good embryo development following in vitro fertilization with frozen-thawed ram semen [59], although no significant difference was found after cervical insemination [3].

Seminal plasma proteins (SPP) are able to protect ram spermatozoa against cold-shock [47,62,63], and, whether added alone or with certain antioxidant compounds, exert a protective effect not only regarding sperm quality parameters but also antioxidant enzyme activity [Superoxide dismutase (SOD), Glutathione peroxidase (GPx), and Glutathione reductase (GR)] in fresh, cooled and frozen/thawed sperm samples [64]. Furthermore, the mixture of SPP, oleic/linoleic acids, and vitamin E was able to partly maintain and recover the enzyme distribution on fresh sperm samples, particularly of SOD [64]. Furthermore, the greater activity of GPx and SOD in seminal plasma was found in two fractions that contain two protein bands, RVSP 14 and RSVP 20, which are responsible for this protective effect [65]. Heat-shock proteins have been identified as components of the oviductal epithelium and of spermatozoa themselves [66-69]. One sperm-binding oviductal protein, the heat shock 70 kDa protein 8 (HSPA8) has been identified using proteomic analyses [70], and the recombinant form of HSPA8 has been shown to support the in vitro survival of boar, bull and ram spermatozoa [71,72]. Therefore, in the current study the protective effect of SPP was tested alone and in combination with HSPA8, a natural sperm protector under physiological conditions [73].

Material and Methods

Animals and samples handling

All the experiments were performed with fresh semen taken from nine mature Rasa Aragonesa rams using an artificial vagina. All the rams belonged to the National Association of Rasa Aragonesa Breeding (ANGRA) and were 2-4 years old. They were housed under uniform nutritional conditions at the Experimental Farm of the University of Zaragoza in compliance with the requirements of the European Union for Scientific Procedure Establishments. All experimental procedures were performed under the supervision of the Ethics Committee of the University of Zaragoza, in accordance with the directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes (published in the Official Journal of the European Union, 20.10.2010, L 276/33). The sires were kept apart, and semen was collected every two days, in two successive matings each day. Under these conditions, and using second ejaculates, individual differences are very low, as we have already reported [74], and pooled ejaculates provide a good quality uniform sperm sample suitable for representative studies of ram semen. For every experiment, the second ejaculates from each group (4 rams) were pooled and processed together.

Semen cooling and freeze-thawing

Semen was diluted to 500 µL (1:5) with a dilution medium (DM) composed of (HEPES 21 mM, glucose 2.7 mM, Sodium pyruvate 1mM, lactate 18.6 mM, sucrose 200 mM, pH 6.5 [75]) containing 5% glycerol, at 30°C within 30 min after collection. Then, sperm quality parameters were assessed in diluted semen (fresh samples), control (with no additive) and treated (with additives) samples.

In the refrigeration experiments, the samples were gradually cooled to 5°C, and at -0.2°C/min using a programmable water bath (total refrigeration process was 2 h 20 min). After further incubation at 5°C for 1 h, the samples were rewarmed up to 37°C in a water bath and kept at this temperature for a further hour. Aliquots of each sample were taken for analysis at 5°C, after 1 h of incubation at 5°C, and at 37°C, and after 1 h of incubation at 37°C.

In the freeze-thaw experiments, the samples were frozen by the pellet method described by Evans and Maxwell [76]. Frozen semen pellets were obtained by placing 200 µL droplets of the cooled diluted semen directly onto small holes made on dry ice for 2 min and then transferred to liquid nitrogen until use, when they were thawed in dry glass tubes (1.5 min at 37°C) and immediately diluted 1:1 in Tris-citrate-fructose medium. For the thawing process, each pellet was placed in a water bath at 37°C in a glass tube and shaken for at least 1.5 min. Semen was diluted 1:2 with DM and incubated for further 6 h in a water bath at 37°C. Sperm quality parameters were assessed immediately after thawing (0 h) and following further incubation for 3 and 6 h at 37°C in a water bath. Each experiment was replicated 3 times.

Additives

In general terms, hydrophilic reagents were directly diluted in the media, controlling pH. Hydrophilic compounds were diluted on a vehicle (PBS: DMSO, 9:1) which was included on the trial. Osmotic differences between media were measured and assumed as no relevant.

All additives were added to DM, prior to semen dilution. Different concentrations of oleic/linoleic acids (25 µM, 40 µM, 50 µM and 75 µM of an equimolar solution containing both molecules, Sigma-Aldrich Quimica, Madrid, Spain) were assayed. The concentration of melatonin used in the refrigeration essays (1 µM and 10 µM, Sigma-Aldrich Quimica, Madrid, Spain) was chosen on the basis of previous results that showed that the exposure of ram spermatozoa to 1 µM melatonin has a direct effect and resulted in decreased capacitated pattern [45]. Pyruvic acid (10 µM and 100 mM, α-ketoprotic acid, Sigma-Aldrich
Quimica, Madrid, Spain) was added to DM containing either 200 mM sucrose (Panreac Quimica S.A., Barcelona, Spain) (normal DM) or 200 mM trehalose (DMt, replacing sucrose by trehalose) (D-(-)-Trehalose dihydrate, Fluka, Sigma-Aldrich Quimica, Madrid, Spain).

Given the possible agonist effect of certain antioxidants, in the freeze-thawing experiments, we also assayed the effect of 4 pairs of compounds associated according to their action mechanism: 25 µM oleic/linoleic acids with 2 mM tocopherol (α-tocopherol phosphate disodium salt, Sigma-Aldrich Quimica, Madrid, Spain); 0.02 mM α- lipoic and 73 mM ascorbic acids (Sigma-Aldrich Quimica, Madrid, Spain); 1 mM or 3 mM melatonin and 10 µM pinoline (6-methoxy-1,2,3,4-tetrahydro-β-carboline, Sigma-Aldrich Quimica, Madrid, Spain); 1.5 mM N-acetyl-L-cysteine (Sigma-Aldrich Quimica, Madrid, Spain) and 5 mM L-glutathione reduced (GSH, Sigma-Aldrich Quimica, Madrid, Spain).

Other compounds used were lecithin (3.5% W/V L-α-phosphatidylcholine from soybean, Type II-S, Sigma-Aldrich Quimica, Madrid, Spain) that was first diluted and then filtered twice trough 22 µM pore filter, and 4 µg/mL HSPA8 (Assay Designs, Grupo Taper S.A, Madrid, Spain) diluted in PBS that was added before freezing.

Seminal plasma was obtained by centrifuging 1 mL of semen at 12000 xg for 5 minutes in microfuge at 4°C. The supernatant was centrifuged again: 400 µL of undiluted seminal plasma were removed and, after filtering through a 0.22 µm membrane, was kept at -20°C. Seminal plasma proteins (SPP) were obtained by filtering the whole seminal plasma through Microsep microconcentrators of 3 kDa molecular weight cut-off (Filtron Tech. MA, USA), spinning for 6 h at 3000 xg at 4°C. The obtained sample concentrated was diluted with 5 volumes of a medium containing 0.25 M sucrose, 0.1 mM EGTA (Sigma-Aldrich Quimica, Madrid, Spain), 4 mM sodium phosphate pH 7.5, 10% (v/v) of "10x buffer stock Heps" (50 mM glucose, 100 mM Heps, 20 mM KOH, Sigma-Aldrich Quimica, Madrid, Spain) and centrifuged again, and SPP were recovered and stored at -20°C. Protein quantification was assessed by the Bradford [77]. The SPP concentration used was 25 mg/mL, based on our preliminary results [62].

Analysis of sperm motility

Total and progressive sperm motility was evaluated by computer-assisted analysis using a CASA system (ISAS, Proiser SL, Valencia, Spain). The hardware was a Basler A312f camera connected to a Nikon Eclipse 50i microscope with negative phase contrast and a 10x objective.

All quots of 8 µL of diluted sample (1/100 as indicated above) were placed on a pre-warmed slide (37°C), covered with a 22 × 22" cover slip, and images were recorded for 1.5 s (25 images/s; at least 200 cells of each slide). The percentage of total motile and progressive motile sperm was analysed in all samples.

Flow cytometry analysis

Stains for viability were CFDA (0.46 mg/mL) and Propidium Iodide (0.5 mg/mL) [78] (Sigma-Aldrich Quimica S.A, Madrid, Spain). 5 µL of each were added to 500 µL of sample with a concentration of 6 × 10⁶ spermatozoa/mL. Three subpopulations were differentiated: CFDA+/PI- (membrane-intact spermatozoa with active esterase, V), CFDA+/PI+ (active esterase but permeable membrane) and CFDA-/PI+ (non-viable).

To assess membrane stability and permeability together with mitochondrial membrane potential (MMP), a triple stain was performed with YoPro1 (1 mM in DMSO; Invitrogen S.A. Barcelona, Spain), PI as indicated above and Mito Tracker Deep red (10 µM in DMSO; Invitrogen S.A. Barcelona, Spain); 5 µL of each were added to 500 µL of sample with a concentration of 6 x 10⁶ spermatozoa/mL that were incubated in the dark for 15 min at room temperature. Data for YoPro1/PI/Mito Tracker were interpreted as described by Hallap et al. [80], regarding spermatozoa with high fluorescence as having a high ΔΨm. Thus, the obtained results are based on the population YoPro1-/PI- and high ΔΨm.

All stained samples were analysed by flow cytometry in a Beckman Coulter FC 500 cytometer. YoPro1, CFDA and PI were excited at 488-nm argon using an ion laser, and Mito Tracker Deep Red was excited at 635-nm using a solid-state laser. From each spermatozoa, forward and side scatter (FSC and SSC, respectively) and the emission of each fluorochrome were recorded using three photodetectors, as follows: FL1 for YoPro1and CFDA (525BP filter); FL3 or FL4 for PI (620BP or 675BP filters) and FL5 for Mito Tracker Deep Red (755LP filter). Acquisition was controlled using CXP software (Beckman Coulter). All parameters were determined using logarithmic amplification. The filtered events were displayed in dot plots showing FL1/FL4 (CFDA v. PI) for viability; FL1/FL3 (YoPro1 v. PI), FL1/FL6 (YoPro1 v. Mito Tracker Deep Red) for the triple stain; and FSC/SSC from both to monitor the correct acquisition of the investigated parameters, and 20,000 events were acquired per sample.

Data processing and statistical analysis

The results are shown as mean ± SEM of three replicates. The data obtained were analysed by the Chi-square test (SPSS Software, v.14.0). When the chi-square test showed significant differences between groups, pairwise comparisons were made using the Fisher’s exact test.

Results

Effect of antioxidants during cooling

With the aim of defining an adequate extender composition, we tested whether the addition of oleic/linoleic acids, pyruvic acid or melatonin to the sperm dilution medium (DM) was able to protect ram spermatozoa during the refrigeration step.

We found no significant beneficial effect of a commercial mix of oleic/linoleic acid at 25 µM, 40 µM, 50 µM and 75 µM on viability (Table 1). However, a higher (p<0.001) value of total motility was found after 1 h at 37°C in the presence of 25 µM, 40 µM, 50 µM and 75 µM (54.2 ± 12.6, 72.7 ± 3.0, 64.2 ± 12.2 and 66.9 ± 4.2%, respectively) compared to control samples (39.4 ± 22.2% (Figure 1A). Likewise, progressive motility was higher (P<0.001) with 75 µM oleic/linoleic acid immediately after rewarming at 37°C, and it even persisted higher (p<0.001) after one further hour of incubation at 37°C in samples containing 25 µM, 40 µM, 50 µM and 75 µM (23.5 ± 5.7, 32.6 ± 2.5, 29.6 ± 6.5 and 36.1 ± 2.9%, respectively vs. 10.7 ± 7.3, Figure 1B).

To determine whether trehalose could be a more efficient protective agent that sucrose, and, at the same time the effect of two different pyruvate concentrations, we added 10 or 100 mM pyruvate to DM, which already contains 1 mM pyruvic acid (control sample) and to DM in which sucrose was replaced with trehalose (DMt). When pyruvic acid was added to DMt, viability values decreased (p<0.001) after reaching...
found.

differences for each time between treatments and control (with no additive) were
refrigerated (5ºC), further incubated at 5ºC for 1 h (5ºC + 1 h), rewarmed up to
37ºC and kept at this temperature for a further hour (37ºC + 1 h). No significant
differences for each time between treatments and control (with no additive) were
found.

Table 1: Sperm viability (%; Mean ± SEM) of refrigerated ram semen with
different concentrations of oleic/linoleic acids (O/L). Diluted samples (Fresh) were
refrigerated (5ºC), further incubated at 5ºC for 1 h (5ºC + 1 h), rewarmed up to
37ºC and kept at this temperature for a further hour (37ºC + 1 h). No significant
differences for each time between treatments and control (with no additive) were
found.

| Variables | Fresh | 5ºC | 5ºC + 1 h | 37ºC | 37ºC + 1 h |
|-----------|-------|-----|----------|------|-----------|
| Control   | 70.5 ± 5.7 | 46.0 ± 2.4 | 40.1 ± 1.4 | 42.9 ± 1.7 | 48.9 ± 3.7 |
| 25 uM (O/L) | 67.0 ± 6.0 | 50.0 ± 4.9 | 45.2 ± 1.8 | 44.3 ± 2.0 | 51.5 ± 1.3 |
| 40 uM (O/L) | 66.9 ± 4.3 | 53.0 ± 5.7 | 44.4 ± 3.7 | 43.2 ± 3.5 | 51.8 ± 2.6 |
| 50 uM (O/L) | 65.3 ± 6.9 | 50.1 ± 4.2 | 45.1 ± 4.4 | 43.0 ± 6.5 | 55.9 ± 1.5 |
| 75 uM (O/L) | 64.8 ± 4.8 | 51.4 ± 4.8 | 42.5 ± 4.4 | 47.1 ± 0.5 | 53.9 ± 0.4 |

Figure 1: Sperm motility (%) of refrigerated ram semen with different
conzentations of oleic/linoleic acids: Total (A) and progressive (B) motility. Significant differences for each time between treatments and control (with no additive) (*p<0.05; **p<0.01; ***p<0.001).

5ºC (Table 2). However, the addition of 10 mM pyruvic acid to DM accounted for the highest values of total motile cell at 5ºC (Figure 2A). Likewise, these samples also showed higher progressive motility values, which were significantly higher after re-warming at 37ºC (Figure 2B).

The addition of melatonin to the semen dilution medium neither resulted in improved viability (Table 3) nor motility (Figures 3A and 3B).

Effect of antioxidants during freeze-thawing

In order to have a good control of the initial state of the spermatozoa, sperm quality parameters were determined in fresh samples (diluted in DM). Mean values of the sperm percentage with integral plasma membrane (Viability measured by CFDA/PI), non-apoptotic membrane (YoPro and PI not stained cells) and integral membrane and functional mitochondria (PI not stained cells stained with MitoTracker) in these fresh samples were (59.8 ± 8.6%, 66.4 ± 9.2% and 57.7 ± 9.6%, respectively). Total and progressive motility was 85.2 ± 2.3% and 41.4 ± 4.7%, respectively.

We determined the effect of melatonin (1 mM and 3 mM), N-acetyl cysteine (Nac, 1.5 mM and 5 mM) and pinoline (10 µM and 100 µM) on frozen-thawed ram semen diluted in DM. No significant differences were found in viability between controls and samples with additives (Table 4). Regarding motility, the addition of 5 mM Nac resulted in a higher (P<0.01) percentage of total (Figure 4A) motile sperm at thawing time, while no significant effect was found in progressive motility (Figure 4B). Both values dropped drastically after 6 h of incubation.

In order to assess the effect of ascorbic acid, we added different concentrations (5, 25, 75 and 125 mM) to DM, and membrane integrity was evaluated simultaneously to mitochondrial functionality as
supplementary information. The obtained results showed a beneficial effect of ascorbic acid with significant increases in viability, membrane stability (YoPro-) and mitochondrial membrane potential (MMP) (Table 5). After thawing, samples supplemented with 75 mM ascorbic acid scored higher proportions of viable, YoPro- and with high MMP cells than those in control samples. This effect persisted even after 3 and 6 h of incubation at 37°C. Additionally, ascorbic acid also had a beneficial effect on post-thaw motility at thawing time (Figures 5A and 5B) with higher (p<0.001) percentages of total motile and progressive sperm with 25 and 75 mM.

Given the possible agonist effect of certain antioxidants, we studied the result of the addition of 4 pairs of compounds to DM, combined according to their action mechanism: oleic/linoleic acids with tocopherol, lipoic and ascorbic acids, melatonin and pinoline, N-acetyl and GSH. We found no significant effect on membrane integrity or MMP (Table 6), and on motility only at thawing time when all additives were used together (Figure 6).

As a different approach, we added lecithin and two types of proteins,
and control (with no additive) were found. Samples were assessed immediately after thawing (0 h) and following further incubation for 3 and 6 h at 37ºC. No significant differences for each time between treatments with a mix of all them (Total). Viability (V) measured by CFDA/PI; YoPro and PI not stained cells (YoPro-); YoPro and PI not stained cells stained with MitoTracker (MT+).

$$1.5 \text{ mM N-acetyl cysteine + 5 mM GSH (Nac/GSH), 0.02 mM lipoic acid + 73 mM ascorbic acid (Lip/Asc), 25 µM oleic/linoleic acids + 2 mM tocopherol (O/L/Toc), and}$$

Table 6: Sperm quality characteristics (%; Mean ± SEM) of frozen-thawed ram semen with different pairs of antioxidants: 1 mM melatonin + 10 µM pinoline (Mel/Pin), 1.5 mM N-acetyl cysteine + 5 mM GSH (Nac/GSH), 0.02 mM lipoic acid + 73 mM ascorbic acid (Lip/Asc), 25 µM oleic/linoleic acids + 2 mM tocopherol (O/L/Toc), and with a mix of all them (Total). Viability (V) measured by CFDA/PI; YoPro and PI not stained cells (YoPro-); YoPro and PI not stained cells stained with MitoTracker (MT+).

Samples were assessed immediately after thawing (0 h) and following further incubation for 3 and 6 h at 37ºC. No significant differences for each time between treatments and control (with no additive) were found.

Similarly, samples containing SPP and lecithin showed the highest motility values. The proportion of total motile sperm was higher (p<0.001) in samples containing SPP and lecithin immediately after thawing. A total motility value of 6.2 ± 1.2% increased up to 35.2 ± 3.2% with SPP, lecithin and HSPA (Figure 7A). Furthermore, SPP and lecithin preserved higher (p<0.001) values 3 h after thawing. Similar results were found in progressive motile cells (Figure 7B), and samples containing SPP and lecithin showed the highest value (p<0.001) at thawing time.

**Discussion**

One of the main drawbacks of refrigerated and frozen-thawed semen is the lipid peroxidation, which results from the collapse of antioxidant enzyme defenses, and the subsequent generation of ROS from cellular metabolism [82]. In order to try to avoid these effects, external antioxidants can be added to extenders.

In this study, we analysed the effect of several compounds in ram semen cryopreservation, trying to maintain good viability during cooling, in order to start the freezing process with adequate sperm quality. Our procedure of cooling semen to 5°C and rewarming to 37°C (incubation in both cases for 1 h) was very useful for revealing differences in the cryoprotective effect of the studied additives, and some conclusions can be inferred. The oleic-linoleic mix improved sperm motility after refrigeration and rewarming. The addition of pyruvate in the presence of a different disaccharide, i.e. trehalose, accounted for decreased viability values compared with the sucrose-containing medium. This result corroborates our previous findings which showed that sucrose is a better option than disaccharides in extenders for ram semen [83].

In the freezing-thawing experiments, the absence of egg yolk or any other source of membrane protectors (i.e., soy lecithin) made the maintenance of motility impossible, and resulted in really low cell functional preservation. The analyses of correlation between motility, viability and other sperm characteristics and in vivo fertility of frozen-thawed semen point to the requirement for adequate cryoprotectors to preserve sperm quality [84,85]. Antioxidants by themselves were unable to provide any egg yolk-like cryoprotection. Because semen was diluted 1.5, we increased the melatonin concentration in the freezing-thawing assays, trying to reach the value that we found in seminal plasma [40], and described as able to prevent protein peroxidation [46]. However, the effect was detrimental to frozen-thawed sperm quality, while samples with Nac scored the best motility results. Therefore, Nac might be a suitable additive for ram semen diluents as it is easily acquired and hydrosoluble, avoiding problems with any vehicle, such as DMSO, needed for dilution of both pinoline and melatonin. Given

**Table 6: Sperm quality characteristics (%; Mean ± SEM) of frozen-thawed ram semen with different pairs of antioxidants: 1 mM melatonin + 10 µM pinoline (Mel/Pin), 1.5 mM N-acetyl cysteine + 5 mM GSH (Nac/GSH), 0.02 mM lipoic acid + 73 mM ascorbic acid (Lip/Asc), 25 µM oleic/linoleic acids + 2 mM tocopherol (O/L/Toc), and with a mix of all them (Total). Viability (V) measured by CFDA/PI; YoPro and PI not stained cells (YoPro-); YoPro and PI not stained cells stained with MitoTracker (MT+). Samples were assessed immediately after thawing (0 h) and following further incubation for 3 and 6 h at 37ºC. No significant differences for each time between treatments and control (with no additive) were found.**
be pointed out.

Although this is a subjective appreciation that didn’t confer a significant difference in any sperm quality parameter, it must be pointed out.

appropriate pH control, another hydroxysoluble and beneficial additive might be ascorbic acid. In this study 75 mM ascorbic acid was able to improve sperm functionality at the level of both the plasma and mitochondrial membranes. The beneficial effect persisted even after 6 h of incubation. These results might be explained by the fact that one stimulus for apoptosis comes from mitochondrial dysfunction, and indicates a possible relationship between mitochondrial protection and the prevention of apoptotic-related changes in the spermatozoa.

Surprisingly, the mix of several antioxidants did not result in any significant effect, which might be due to the lack of any membrane protector. However, it is worth noting that 6 h after rewarming, we observed (in motility studies) that certain antioxidants provided different features to the sperm membrane, induced cell aggregation and conferred a ‘dirty’ aspect to the samples, while others like Nac/GSH maintained transparent media and ‘clean’ cells without aggregation (data not shown). Although this is a subjective appreciation that didn’t confer a significant difference in any sperm quality parameter, it must be pointed out.

Our results also revealed curious differences between the staining data. The percentage of cells stained with PI was not the same in both cases, CFDA/PI and YoPro1/PI/Mito Tracker, although it could be expected. CFDA/PI staining is stricter and, in general, resulted in a lower proportion of viable cells than that obtained with YoPro1/PI/Mito Tracker. This difference might be due to the fact that CFDA stains cells not only with impermeable membrane but also with active esterases [78], while YoPro1 stains cells with destabilized plasma membrane. Although not all stable membranes have their esterase active, a significant correlation between plasma membrane stability and motility has been reported [86]. Furthermore, YoPro1 is only permeant in cells that are beginning to undergo apoptosis [87] and, thus, permits analysis of apoptotic cells without interfering with cell viability [88]. The possibility that YoPro1 and Mito Tracker might either interfere with PI staining or even produce certain membrane impermeability cannot be ruled out. It is worth pointing out that alterations in the inner mitochondrial membrane are reflected in low motility in a time-dependent manner after thawing [89]. Furthermore, a significant correlation between viable sperm with high both MMP and motility [90] and high fertility [91] has been shown. Therefore, the triple combination of the fluorophores YoPro1/PI/Mito Tracker can be useful for accuracy monitoring the functionality of frozen-thawed ram spermatozoa.

It has been described that the addition of lecithin resulted in increased proportions of viable and non-apoptotic spermatozoa in fresh and frozen-thawed ram semen [6,57]. However, we also found that lecithin induced certain mitochondrial membrane alterations, which might be due to a decrease in cardiolipin content, that is not reflected in sperm motility evaluated immediately after thawing but along with thawing time [89]. This fact might explain why no significant effect of lecithin was found after intrauterine insemination with frozen-thawed semen [6] although good embryo development was shown following in vitro fertilization [59].

Accordingly, in the present study, the addition of lecithin accounted for increased cell viability, membrane stability and MMP, but the alterations induced in the inner mitochondrial membrane resulted in a huge lower proportion of functional mitochondria in a time-dependent manner after thawing, that may negatively affect the sperm fertilizing capacity. Our results revealed that SPP, which showed a remarkable beneficial effect, prevented the lecithin detrimental effects. Although viability values were very low, the protective effect of lecithin was very strong (552% of increase at 0 h), which is even higher that that found when Salomon's medium was used as a control [89]. This low viability must be due to the low viability values in the control samples, which indicate that DM is not a suitable medium for ram semen
The results of this study showed that certain antioxidants could be useful in the absence of cryoprotectants for cooling ram semen, particularly efficient to preserve motility. The combination of lecithin and SPP could be an appropriate substitute for egg yolk for ram semen cryopreservation.

The data presented in this paper could help in the formulation of better diluents and open new interesting perspectives to cryopreservation. The combination of lecithin and SPP could be useful in the absence of cryoprotectants for cooling ram semen, particularly efficient to preserve motility. The combination of lecithin and SPP could be an appropriate substitute for egg yolk for ram semen cryopreservation.

The addition of HSPA8 did not result in any significant improvement, even when supplemented with other compounds. Therefore, we infer that their ability is more related with prolonging lifespan than protecting cells from cryodamage. The potential role of HSPA8 on maintaining sperm quality has been reported in Brown bear frozen-thawed semen, although no significant improvement was found immediately after thawing, only after a 2-hour incubation stress test. In that study, not only was the extender composition different (it included egg yolk) but also the HSPA8 concentration used was lower (1 μg/ml). Our cryopreservation data do not support previous reports that described the sperm survival promoting effects of HSPA8 in ram, boar and bull semen. However, in those studies the spermatozoa were co-incubated with HSPA8 over an extended period at 39°C and not subjected to cryopreservation. Furthermore, the difference might also be due to alterations in the sperm surface properties between samples, because in this study, we used raw semen, while in the other experiments the semen was either washed through Ficoll or Percoll gradients, or centrifuged and washed. The influence of the processing that semen has previously suffered on the seminal plasma effect has already been reported. Moreover, semen treatments not only select a higher density sub-population of spermatozoa but also induce the removal of proteins and/or other cell surface-coated components, similar to the decorating that the sperm surface suffers during the transport in the female tract. The induced sperm surface modifications might facilitate the adsorption of HSPA8.

Conclusion

The addition of HSPA8 did not result in any significant improvement, even when supplemented with other compounds. Therefore, we infer that their ability is more related with prolonging lifespan than protecting cells from cryodamage. The potential role of HSPA8 on maintaining sperm quality has been reported in Brown bear frozen-thawed semen, although no significant improvement was found immediately after thawing, only after a 2-hour incubation stress test. In that study, not only was the extender composition different (it included egg yolk) but also the HSPA8 concentration used was lower (1 μg/ml). Our cryopreservation data do not support previous reports that described the sperm survival promoting effects of HSPA8 in ram, boar and bull semen. However, in those studies the spermatozoa were co-incubated with HSPA8 over an extended period at 39°C and not subjected to cryopreservation. Furthermore, the difference might also be due to alterations in the sperm surface properties between samples, because in this study, we used raw semen, while in the other experiments the semen was either washed through Ficoll or Percoll gradients, or centrifuged and washed. The influence of the processing that semen has previously suffered on the seminal plasma effect has already been reported. Moreover, semen treatments not only select a higher density sub-population of spermatozoa but also induce the removal of proteins and/or other cell surface-coated components, similar to the decorating that the sperm surface suffers during the transport in the female tract. The induced sperm surface modifications might facilitate the adsorption of HSPA8.

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