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Title: Post-thawing quality and incubation resilience of cryopreserved ram spermatozoa are affected by antioxidant supplementation and choice of extender

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Abstract

The performance of cryopreserved semen in ovine artificial insemination still needs improvement. Some antioxidants have been tested, with variable success. We cryopreserved semen from Churra rams using TES-Tris-fructose with 4% glycerol and 10% egg-yolk (EY) or 3.5% soybean lecithin (SL), with 1 mM of reduced glutathione (GSH), Trolox, crocin or cysteamine. Samples were analyzed post-thawing and post-incubation (6 h, 38 ˚C) for motility (CASA), viability, acrosomal integrity, apoptosis, mitochondrial activity, chromatin status and lipoperoxidation (malondialdehyde —MDA— production). Interactions (antioxidant/extender/incubation) were significant for most variables. Extenders yielded similar results, although SL depressed mitochondrial activity and linearity ($P<0.001$), but it improved motility ($P<0.05$), DNA fragmentation ($P<0.05$) and acrosomal damage ($P<0.001$). The Control, GSH and Trolox showed greater viability with SL ($P<0.01$). Cysteamine depressed motility (0 h: 51.6%±2.0 vs. 32.3%±4.3; 6 h: no motility vs. 32.5%±1.9; $P<0.001$), but improved viability when using EY ($P=0.004$). Crocin increased acrosomal damage ($P=0.022$), but improved linearity-related parameters post-thawing ($P=0.014$). Trolox considerably reduced MDA production in both extenders (8.6±0.4 nmol per 10^8 cells vs. 14.2±0.3 in EY and 20±0.6 in SL; $P<0.001$). Interestingly, thiol antioxidants (cysteamine and GSH) increased DNA fragmentation (%DFI), whereas crocin reduced it ($P<0.05$). Interactions between extender and antioxidant must be taken into account for improving sperm cryopreservation. Soybean lecithin seems to be a suitable replacement for egg yolk, but its effect on mitochondria must be investigated. Trolox and crocin might be useful for ram semen freezing.

Keywords: ram, spermatozoa, antioxidants, cryopreservation, extender

1. Introduction

The use of semen cryopreservation and its application by conventional artificial insemination (AI) in sheep has been a long standing objective for researchers and practitioners, but reports indicate a very high variability of fertility results when using cryopreserved semen in conventional AI [1]. For instance, some breeds yield good and consistent results, whereas others perform poorly [2]. Improving sperm quality after cryopreservation could help to improve overall results and to diminish the influence of other factors. However, sperm quality is severely affected by current cryopreservation protocols, which affect the structure and resiliency of spermatozoa [3], and make the improvement of these protocols a priority [4].

Cryopreservation subjects the spermatozoa to a series of physical and chemical insults, such as the attack of free radicals, which decreases the viability and fertility of spermatozoa [5]. Spermatozoa are especially vulnerable to the propagation of lipid peroxidation, due to the abundance of polyunsaturated fatty acids (PUFA) [6]. The spermatozoon is protected by an antioxidant system in the seminal plasma, in the membranes and in the cytoplasm, but this system is partly removed and severely altered during cryopreservation [7]. This fact is the foundation of strategies consisting of the supplementation of extenders before freezing [7–12] of after thawing [13].

In this study we have tested the efficacy of the antioxidants reduced glutathione (GSH), Trolox, cysteamine and crocin in cryopreserving ram semen. We chose these antioxidants because of their differences in chemical structure and biological activity, and, in some cases, because of controversial results in previous studies. For instance, Trolox, a water-soluble vitamin E analogue, has been reported to improve post-thawing quality of ram semen [9, 12], but it yielded mixed results when we tested it in red deer spermatozoa [14–17]. Cysteamine and GSH have also resulted in conflicting outcomes when freezing ram semen [8, 18–20]. Nevertheless, we obtained some positive effects in the storage of cooled ram semen when the extender was supplemented with GSH [21], and therefore we included these thiol antioxidants in this trial.

We also tested crocin, a glucosyl ester of crocetin (a carotenoid extracted from saffron, *Crocus sativus*). Crocin has been tested in spermatozoa just once [15], being added to red deer spermatozoa post-thawing. It enabled a considerable improvement for a considerable improvement of sperm motility,

although it also increased lipoperoxidation. These interesting results and the fact that carotenoids have not been tested for sperm cryopreservation (except for a study in poultry [22]), led us to include crocin in this study.

Finally, we aimed at testing the interaction of the antioxidants with extenders differing in the lipid source: egg yolk and soybean lecithin. Extenders could also affect the efficiency of supplements, due to interactions of the different components and to the fact that egg yolk and lecithin may contribute to the total antioxidant activity [23, 24]. Whereas egg yolk has been extensively used as a cryoprotectant, many studies have focused on other options, especially soybean lecithin. Soybean lecithin avoids the use of products of animal origin, preventing microbial contamination, and helps to attain standardization, since egg yolk composition can vary considerably between batches [25].

Therefore, we have tried the antioxidants GSH, Trolox, cysteamine and crocin as supplements of ram sperm extenders to determine their effect on post-thawing quality and on the resilience of thawed spermatozoa, tested after incubation. We combined them with two different cryoprotectants, egg yolk and soybean lecithin, in order to study the interactions of the antioxidants with each cryoprotectant.

2. Materials and Methods

2.1. Reagents and media

Mitotracker Deep Red and YO-PRO-1 iodide were purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals such as propidium iodide (PI), peanut agglutinin conjugated with fluorescein isothiocyanate (PNA-FITC), DMSO and extender components were purchased from Sigma (St. Louis, MO, USA). Stock solutions of fluorochromes were prepared in DMSO at 1 mM, except YO-PRO-1 (75 μM), PI (1 mg/mL in water) and PNA-FITC (0.2 mg/mL in water). These stocks were kept at -20 °C and in the dark. Flow cytometry equipment, software and consumables were purchased from Becton Dickinson (San Jose, CA, USA).

2.2. Experimental design

Semen was collected and subjected to initial assessment. Each sample was split into two aliquots, which were extended with the same volume of TES-Tris-fructose media containing either egg yolk (10%) or

soybean lecithin (3.5%), with 4% glycerol, and cooled down to 5 ˚C. Then, each sample was extended to 100×10^6 spermatozoa/mL with the same media, and split between five tubes. One was left as the Control, and the other received 1 mM of either reduced glutathione, Trolox, crocin or cysteamine. Semen doses were prepared and frozen. After thawing, part of each sample was assessed and the rest was incubated for 6 h at 38 ˚C, and then analyzed. The evaluation consisted of: motility (CASA), acrosomal status (PNA-FITC/PI), membrane permeability and mitochondrial activity (YO-PRO-1/PI/Mitotracker deep red), and chromatin status (SCSA). The experiment was replicated ten times with individual samples from ten rams.

2.3. Semen collection and cryopreservation

We used ten Churra breed adult males (2–5 years old) of proven fertility and trained for semen collection by artificial vagina. The rams were housed in an AI center (Diputación de León, Spain) complying with Spanish and European regulations. Semen collection was performed during the breeding season (autumn). Ejaculates were collected by artificial vagina (water at 40 ˚C), and the tubes were kept in a water bath at 35 ˚C during the initial evaluation of semen quality: volume, mass motility (assessed by microscopy with a warming stage at 37 ˚C, ×40; score: 0–5), and sperm concentration (assessed by the photocolorimetric method at 540 nm, using a specifically calibrated scale). Only ejaculates of good quality were used (volume: ≥0.5 mL; mass motility: ≥4; sperm concentration: $\geq3000\times10^6$ spermatozoa/mL).

The extenders were prepared with 224 mM TES, 85 mM Tris and 12,8 mM fructose (320 mOsm/kg, pH 7.2) in Milli-Q water. One of the extenders (EY) was supplemented with 10% clarified egg yolk (v/v) and the other (SL) was supplemented with 3.5% soybean lecithin (w/v). Both extenders contained 4% glycerol (v/v).

Samples were split in two tubes and extended with the same volume of each extender at ambient temperature, the tubes were placed in a glass with 50 mL of water and transferred to a room at 5 ˚C, where the temperature decreased steadily for two hours. Then, the samples were extended with the respective extender down to 200×10^6 spermatozoa/mL, and distributed among ten tubes (five tubes per extender). One tube per extender received 1 mM of each antioxidant (GSH, Trolox, crocin and cysteamine), the last tube being left as the Control. Semen was packed in 0.25 ml French straws and

equilibrated for 1 h at 5 ˚C and then frozen using a programmable biofreezer (Kryo 10 Series III; Planer plc., Sunbury-On-Thames, UK) at a rate of -20 ˚C/min down to -100 ˚C. The straws were kept in liquid nitrogen containers for at least a year, and thawing was carried out in a water bath at 65 ˚C for six seconds.

2.4. Motility analysis

Motility analysis was performed using the CASA software ISAS v. 1.2 (Integrated Semen Analyser System; Proiser, Valencia, Spain). Samples were diluted in PBS (10–20×10^6 cells/mL) and warmed to 37 ˚C on a plate. Then, a 5 μL drop was placed in a Makler counting chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel) and examined at ×10 (negative phase contrast) in a microscope with a warmed stage (37 ˚C). The standard parameter settings were set at 25 frames/s; 20 to 90 μm^2 for head area and VCL >10 μm/s to classify a spermatozoon as motile. From each sample, images were saved and analyzed afterwards. The software rendered the percentage of motile spermatozoa and standard CASA kinematic parameters: VCL (velocity according to the actual path), VSL (velocity according to the straight path), VAP (velocity according to the smoothed path), LIN (linearity), STR (straightness, WOB (wobble), ALH (amplitude of the lateral displacement of the sperm head) and BCF (head beat-cross frequency).

2.5. Assessment of sperm physiology by flow cytometry

Several physiological traits were assessed by using fluorescent probes and flow cytometry, which were described previously [26]. Samples were diluted down to 10^6 spermatozoa/mL in PBS and stained using the fluorophore combinations PI/PNA-FITC for studying viability and acrosomal status and YO-PRO-1/PI/Mitotracker deep red for studying membrane permeability and mitochondrial status. PNA-FITC was used at 100 μg/mL, PI at 6 μM, YO-PRO-1 at 0.1 μM and Mitotracker Deep Red at 0.1 μM. In all cases, Hoescht 33342 was added at 5 mM to discriminate debris. Spermatozoa stained in these two solutions were incubated for 15 minutes in the dark before being analyzed by flow cytometry.

2.6. Sperm chromatin structure assay

Chromatin stability was assessed following the SCSA (Sperm Chromatin Structure Assay), as performed previously with ram semen [27]. Acridine orange (AO) fluorescence shifts from green (dsDNA) to red

(ssDNA) depending on the degree of DNA denaturation. Samples were diluted in TNE buffer to a final sperm concentration of 2×10^6 cells/mL, and stored at -80 ˚C. For analysis, the samples were thawed on crushed ice and subjected to acid-induced denaturation of DNA in situ and staining with acridine orange. A volume of 200 μL of sample was pipetted in a flow cytometry tube, and it was immediately mixed with 1.4 mL of the acid-detergent solution. After 30 s, 1.2 mL of the acridine orange solution was added to the tube. The tube was kept on ice 3 min before flow cytometry analysis.

2.7. Flow cytometry analyses

Flow cytometry analyses were carried out with a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA), with semiconductor lasers emitting at 405 nm (violet; Hoechst 33342), 488 nm (blue; YO-PRO-1, FITC, PI, acridine orange), and 635 nm (red; Mitotracker Deep Red). Filters used for each fluorochrome were 450/50 (blue) for Hoechst 33342, 530/40 (green) for YO-PRO-1 and FITC, 613/20 (red) for PI, and 665/20 for Mitotracker Deep Red. The system and event analyses were controlled using the Summit software provided with the cytometer. All the parameters were read using logarithmic amplification. For each sample, 5000 spermatozoa were recorded, saving the data in flow cytometry standard (FCS) v. 3 files. The analysis of the flow cytometry data was carried out using WEASEL v. 3 (WEHI, Melbourne, Australia). The PI/PNA-FITC and YO-PRO-1/PI/Mitotracker Deep Red combinations were analyzed as previously described [26, 28].

For the analysis of SCSA samples [29], we used a FACScalibur flow cytometer (Becton Dickinson) and CellQuest v. 3 software for adquisition control. We analyzed 5 000 events per sample, exciting the acridine orange with the Ar-ion 488 nm laser and using a 530/30 filter for the green fluorescence of DNAds-bound acridine orange (AO), and a 650 long pass filter for the red fluorescence of DNAss-bound AO. Data was saved in flow cytometry standard (FCS) v. 2 files, which were processed using the R statistical environment. We calculated the DNA Fragmentation Index (DFI) for each spermatozoon as the ratio of red fluorescence with total fluorescence (red+green). From the DFI values we obtained the percentage of spermatozoon with a high fragmentation index (%DFI, DFI>25%), and the percentage of spermatozoa with high DNA stainability (HDS), defined as those events with green fluorescence above channel 600.

168 *2.8. Statistical Analysis*

169 Data were analyzed in the R statistical environment [30]. We used linear mixed-effects models to analyze
170 the effects of time, extender and antioxidant supplementation (fixed effects), and their interactions. Male
171 was used as the grouping factor in the random part of the models. Results are shown as mean±SEM of
172 the ten replicates.

173 **3. Results**

174 *CASA parameters*

175 Results of the CASA analysis are summarized in Figure 1. Total motility decreased with the incubation
176 (Fig. 1a; $P<0.001$). It was considerably depressed by cysteamine (32.3%±4.3 at 0 h, and no motility was
177 detected in most samples after the incubation), whereas its average value kept above 30% in the rest of
178 the antioxidants (0 h: 51.6%±2.0; 6 h: 32.5%±1.9; $P<0.001$ comparing with cysteamine). Progressivity
179 (Fig. 1b) yielded similar trends and it was also affected by the extender (EY yielding higher progressivity;
180 $P<0.001$).

181 Consequently, VCL and VSL were also reduced when cysteamine was added. It considerably
182 decreased VCL at 0 h (Fig. 1c; $P<0.001$), and in fact no data were available for 6 h, because of the lack
183 of motile spermatozoa in the samples. However, there were no significant differences between the
184 antioxidants and the Control for VSL at 0 h. Considering the extender, SL yielded higher VCL at 0 h
185 ($P=0.005$), but it decreased with time ($P<0.001$), being similar to EY at 6 h ($P=0.771$). Contrarily, VSL
186 was slightly higher for EY overall, and it decreased with time in both cases ($P<0.001$, greater drop for
187 SL).

188 Linearity and wobble (figures 1e and 1f) increased post-thawing in the samples treated with
189 cysteamine ($P<0.001$), and, to a lesser extent in those frozen with crocin ($P=0.014$). Both variables
190 decreased with incubation ($P<0.001$), when crocin became similar to the other antioxidants, whereas
191 cysteamine (almost no data because of low motility) was lower ($P<0.001$). The effect of the extender was
192 also significant, with EY showing higher overall linearity and wobble ($P<0.001$).

193 *Analysis of sperm physiology by flow cytometry*

194 Interactions between the extender with time and antioxidant affected the proportion of viable
195 spermatozoa (Fig. 2a). Viability was higher in the soybean lecithin extender at both times (0 h: $P<0.001$;
196 6 h: $P=0.003$), although the decrease with time was more pronounced in this extender ($P<0.001$ for both
197 extenders). Interestingly, cysteamine had a significantly positive effect on viability when analyzed
198 post-thawing in the EY extender, but not in the SL extender ($P=0.004$). Results for cysteamine and crocin
199 were not significantly different between extenders, whereas for the rest of the antioxidants viability was
200 higher for SL (Control $P=0.002$; GSH $P=0.003$; Trolox $P<0.001$).

201 Acrosomal damage (Fig. 2b) increased with incubation ($P<0.001$) in all cases, from an overall
202 mean of 28.0%±1.2 to 34.9%±1.1 after the incubation. Crocin yielded the highest damage overall
203 ($P=0.022$ vs. Control). Contrarily, the proportion of spermatozoa with increased membrane permeability
204 (YO-PRO-1) within the subpopulation of viable spermatozoa (PI-), termed the apoptotic ratio (Fig. 2c),
205 was not affected by the antioxidant treatment. After thawing, this variable was lower for SL ($P<0.001$),
206 increasing significantly with time ($P<0.001$) and yielding a non-significantly different average value after
207 the incubation. The apoptotic ratio of EY was not significantly affected by time. The mitochondrial
208 activity (Fig. 2d) was affected by a double interaction of incubation time with the extender and
209 antioxidant. Incubation time decreased the proportion of spermatozoa with active mitochondria
210 ($P<0.001$). Cysteamine yielded a higher value just after thawing ($P<0.001$ vs. Control), but it also
211 caused a faster decrease of this variable, and after the incubation it yielded a lower average value than the
212 Control ($P=0.041$). Regarding the extender effect, SL not only yielded a lower value at both times
213 ($P<0.001$), but it also induced a faster decrease during the incubation, increasing the average difference
214 from 7.7±1.6 to 15.1±1.0.

215 *Sperm lipoperoxidation*

216 Sperm lipoperoxidation (Fig. 3a), measured as MDA production, was significantly affected by all the
217 factors and by an interaction between the extender and the antioxidants, caused by a strong inhibition of
218 lipoperoxidation by Trolox. MDA decreased slightly after the incubation with respect to the post-thawing
219 values (overall, 15.6±0.3 to 14.7±0.5 nmol per 10^8 cells, $P=0.043$). MDA was lower in EY for all the

antioxidants except Trolox. Removing Trolox data, EY yielded 14.2±0.3 vs. SL with 20±0.6 nmol per 10^8 cells (P<0.001). Trolox yielded 8.6±0.4 nmol per 10^8 cells overall (P<0.001).

Analysis of sperm chromatin by flow cytometry

The standard deviation of DFI values (SD-DFI, Fig. 3b) was affected by interactions of the antioxidant treatment with time and extender. Time significantly increased this variable in all the treatments. Cysteamine yielded the highest SD-DFI values at both times (P<0.001 vs. Control). GSH increased this variable only after the incubation (P<0.001), whereas crocin achieved a lower value than the Control (P=0.029). We did not detect any significant differences between both extenders within each antioxidant. Cysteamine showed the highest values in both extenders (P<0.001 vs. Control), whereas GSH yielded significantly higher values only when combined with SL (P=0.005).

The proportion of spermatozoa with DFI>25% (%DFI) was affected by all the factors and the interaction time×extender (Fig. 3c). Post-thawing %DFI (in Control samples) was low, below 5% in 6 of the males, between 5% and 15% in three males, and around 23% in one male. After the incubation, only three males remained below 30%, with the rest ranging from 44% to 74%. Cysteamine (P=0.002) and GSH (P=0.017) showed overall higher values than the Control, whereas crocin achieved lower values (P=0.016). At 0 h, the average difference of %DFI between EY and SL was small but significant (P=0.030), but during the incubation it increased more for EY (P<0.001 vs. SL). The proportion of spermatozoa with "immature" chromatin (%HDS, Fig. 3d) was affected by the interaction between time and extender. %HDS was moderate in most cases (values below 10% in all samples). Its average value was lower after the incubation, and EY showed a lower value at both times (P<0.001).

4. Discussion

We have tested four antioxidants for freezing ram semen, combining them with two lipidic cryoprotectants. Some antioxidants such as crocin and Trolox, moderately enhanced some quality variables, whereas cysteamine exerted mostly negative effects. The extenders also showed different effects on many variables, with egg yolk and lecithin significantly affecting the outcome of the cryopreservation.

The choice of cryoprotectant is critical for the success of sperm freezing. Egg yolk has been the preferred choice for supplementing extenders in many species. However, egg yolk could present contamination [31], some components could negatively affect sperm cryopreservation [32] and standardization is challenged by between-batch variability [25]. The use of soybean lecithin could prevent some of these problems, and it could yield results comparable to egg yolk [33–36].

Nevertheless, other authors have found some detrimental effects of soybean lecithin on ram semen, such as decreasing mitochondrial activity Del Valle et al. [37]. Our results support these findings. We also obtained similar results when semen was extended with 3.5% soybean lecithin for refrigerated storage [21]. The drop in mitochondrial potential has been associated to the activation of apoptotic pathways, mediated by the release of cytochrome c from the mitochondria [38]. However, neither Del Valle et al. [37] nor ourselves were able to detect an increase in apoptotic features or even a decrease in viability proportional to the loss of mitochondrial activity. Indeed, viability was higher in the samples frozen with soybean lecithin, and motility was not affected, agreeing with previous reports [35, 39–41]. Nevertheless, Del Valle et al. [37] detected a drop in motility concomitantly with the mitochondrial activity loss. In many species, motility could be supported mainly by glycolysis, being largely independent from the mitochondrial status [42–44], and therefore explaining why our SL samples maintained high motility even after the 6 h incubation.

Soybean lecithin protected sperm chromatin more efficiently than egg yolk. A previous study found that this cryoprotectant had a relatively high antioxidant capacity [24]. Therefore, the lower degree of chromatin damage and other beneficial effects, such as the lower proportion of damaged acrosomes, could be due to this greater antioxidant effect. This contrasts with the increased malondialdehyde yield of these samples. A possible explanation is that part of the malondialdehyde would originate from the oxidation of some lecithin components, instead of from the sperm membranes.

Antioxidants exerted very different effects. Even cysteamine and GSH, which are based on the activity of a thiol group, showed several differences. The effect of cysteamine was unexpected, since it has been reported to be adequate for freezing ram and goat semen[8, 45]. A recent study [18] found an inhibition of motility using 5 mM cysteamine for freezing ram semen. It is not clear why cysteamine produced such different results in studies using similar methods. We also detected effects on motility and

mitochondrial activity (modulated by extender and incubation time) and a clear increase of DNA fragmentation. While the increase in %DFI could be caused by the reducing effect of the thiol group, which might contribute to chromatin decondensation, it is not evident why cysteamine affects mitochondrial activity and motility. The activation of apoptotic pathways or the increase in free radicals seems to be unlikely, according to the apoptotic ratio and lipoperoxidation results. As a hypothesis, the small size of this molecule could allow it diffusing quickly throughout the cell, affecting the redox balance and affecting protein function by reducing disulphide bonds.

GSH supplementation, despite of good results in other studies [20, 46, 47], was unable to improve sperm quality above control levels. There are conflicting results in the literature, with several studies reporting no effects or even negative ones [19, 20, 48]. Our results contrast with a previous study in red deer [17], in which 1 and 5 mM of GSH improved motility and mitochondrial activity, especially after incubating the samples for 6 h at 39 ˚C. Interestingly, in this particular study 1 and 5 mM of Trolox exerted a detrimental effect, which was not observed in the present study. We have to take into account that we used epididymal spermatozoa in our studies with red deer, which might respond differently to these antioxidants. However, ram ejaculated spermatozoa and deer epididymal spermatozoa yielded similar results in other experiments [13, 49].

Similarly to cysteamine, GSH increased %DFI (also observed in [17]). This effect could be due to GSH participating in the reduction of the disulfide bonds of the protamines, therefore relaxing the sperm nucleus and increasing %DFI, but this hypothesis does not explain why the %HDS parameter, which is related to chromatin compactness, was not affected. Contrarily, GSH has a protective effect on boar sperm chromatin [47], and even a stabilizing effect on disulfide bonds [50], possibly because of the different structure of the nucleus in different species.

Trolox was the only antioxidant that reduced lipoperoxidation, almost halving malondialdehyde levels with respect to our control. Trolox has proven to block sperm lipid peroxidation in other studies [9, 15, 17]. In fact, Trolox remediated the increased malondialdehyde production in samples frozen with lecithin. Thus, its capacity to abolish lipoperoxidation could make it a suitable component of optimized extenders, maybe at lower concentrations [15].

Crocin has showed contradictory effects when applied in thawed red deer spermatoza [15]. In that

study it improved sperm motility, but it also increased intracellular free radicals and lipid peroxidation. However, when oxidants were added, it decreased free radicals and DNA damage. In our study, crocin did not improve sperm motility, but it protected sperm DNA. Another carotene, lycopene, prevented human sperm DNA fragmentation at 5 μM [51]. Despite the differences between species and experimental designs, carotenoids could be an interesting option for supplementing sperm media, deserving further research.

The interactions found in this study highlight the complexity of designing a good cryopreservation extender. The effects of supplements, such as antioxidants, are modulated by other extender components of the extenders, and possibly by other parameters such as cooling rate, equilibration time, or sample source. Our results suggest that antioxidants might compensate for some of the weakness of the extenders. It is necessary to investigate these interactions at the molecular level, in order to guide future empirical approaches. In general, soybean lecithin appears to be a suitable replacement for egg yolk, but future research should focus on its effects in mitochondria. Considering fertility results from previous studies, it seems that this effect on mitochondrial status does not depress fertility proportionally, but it might be important when spermatozoa are used at lower concentrations or for other artificial reproduction techniques.

Our results indicate that cysteamine would not be suitable as a supplement for freezing ram semen, although the results reported here could be a consequence of interactions with our cryopreservation protocol, resulting in good results in other conditions. GSH altered sperm chromatin, but it might be useful at lower concentrations and for specific samples. Trolox and crocin could be beneficial for freezing ram semen, especially for improving specific protocols.

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479 FIGURE LEGENDS

480 FIGURE 1

481 Results of the CASA analysis (mean±SEM). Groups are Antioxidants (one panel) or Antioxidant and
482 Extender (two panels) vs. Time, depending on the significance of the factors as main effects or
483 interactions. The significant effects of each model are shown in the insets. When interactions are not
484 significant, asterisks beside an antioxidant indicate that $P < 0.05$ vs. the Control as a main effect (VCL).
485 When interactions are significant, asterisks above or below an antioxidant indicate that $P < 0.05$ vs. the
486 Control, within that time. Different Latin letters above or below each extender indicate $P < 0.05$ between
487 the extenders within that time, and different Greek letters indicate $P < 0.05$ between times within that
488 extender.

489 FIGURE 2

490 Results of the flow cytometry analyses of sperm physiology (mean±SEM). Groups are Antioxidants (one
491 panel) or Extender (second panel for Mitochondrial Activity) vs. Time, or Antioxidants vs. Extender
492 (second panel for Viability), depending on the significance of the factors as main effects or interactions.
493 The significant effects of each model are shown in the insets. When interactions are not significant,
494 asterisks beside an antioxidant indicate that $P < 0.05$ vs. the Control as a main effect. When interactions
495 are significant, asterisks above or below an antioxidant indicate that $P < 0.05$ vs. the Control, within that
496 time. Different Latin letters above or below each extender indicate $P < 0.05$ between the extenders within
497 that time or antioxidant, and different Greek letters indicate $P < 0.05$ between times within that extender.

498 FIGURE 3

499 Results of the analyses on lipoperoxidation and chromatin status (mean±SEM). Groups are Extender
500 (one panel) or Antioxidant and Extender (two or three panels) vs. Time, or Antioxidants vs. Extender
501 (third panel for Liporeroxidation and SD-DFI), depending on the significance of the factors as main
502 effects or interactions. The significant effects of each model are shown in the insets. When interactions
503 are not significant, asterisks beside an antioxidant indicate that $P < 0.05$ vs. the Control as a main effect.
504 When interactions are significant, asterisks above or below an antioxidant indicate that $P < 0.05$ vs. the

505 Control, within that time or extender. Different Latin letters above or below each extender indicate P <

506 1.5 between the extenders within that time, and different Greek letters indicate P < 0.05 between times

507 within that extender.

FIGURE 1

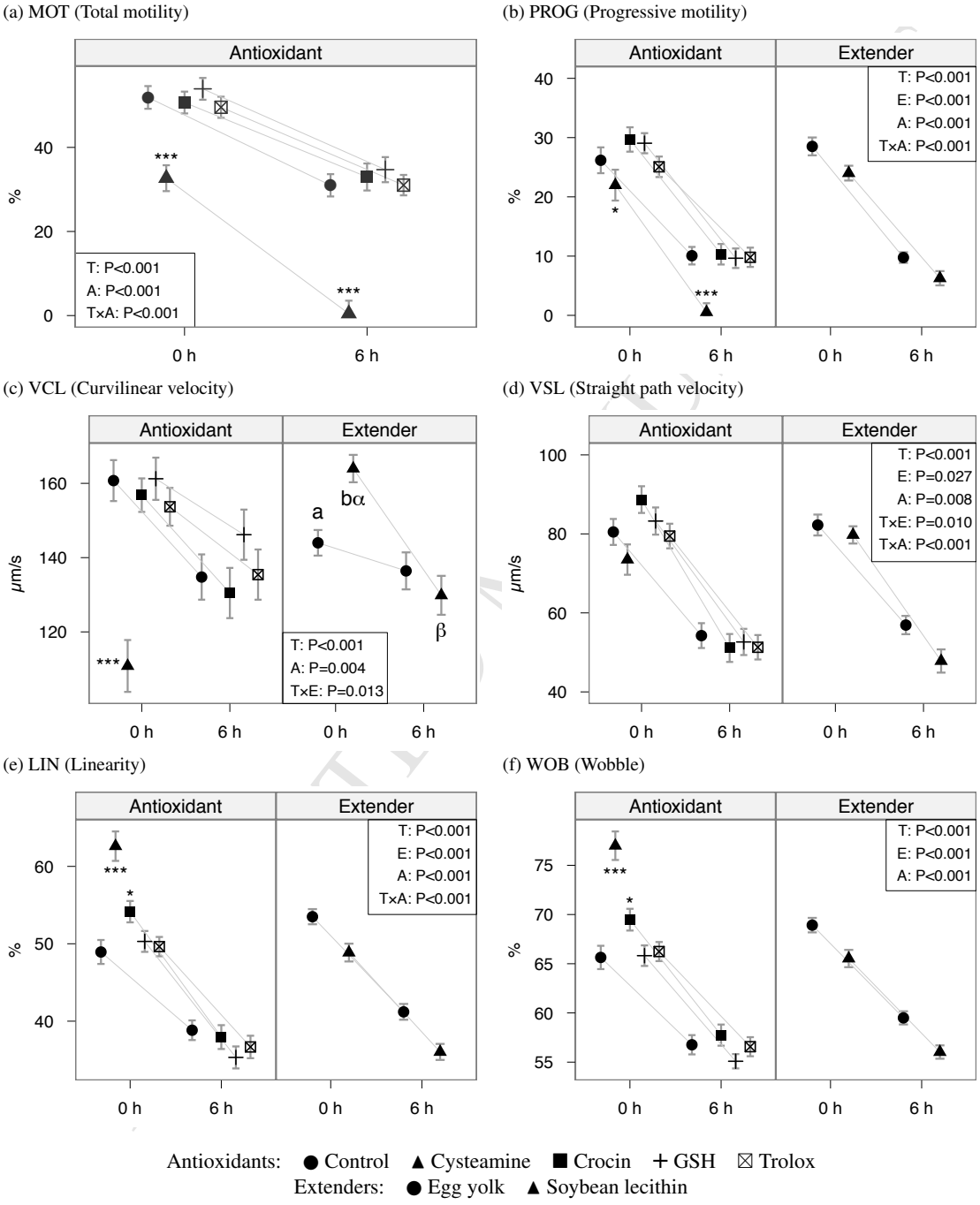

(a) MOT (Total motility)
Antioxidant
%
T: P<0.001
A: P<0.001
TxA: P<0.001
0 h
6 h
(b) PROG (Progressive motility)
Antioxidant
Extender
T: P<0.001
E: P<0.001
A: P<0.001
TxA: P<0.001
(c) VCL (Curvilinear velocity)
μm/s
T: P<0.001
A: P=0.004
TxE: P=0.013
(d) VSL (Straight path velocity)
T: P<0.001
E: P=0.027
A: P=0.008
TxE: P=0.010
TxA: P<0.001
(e) LIN (Linearity)
(f) WOB (Wobble)
Antioxidants: ● Control ▲ Cysteamine ■ Crocin + GSH ☒ Trolox
Extenders: ● Egg yolk ▲ Soybean lecithin

FIGURE 2

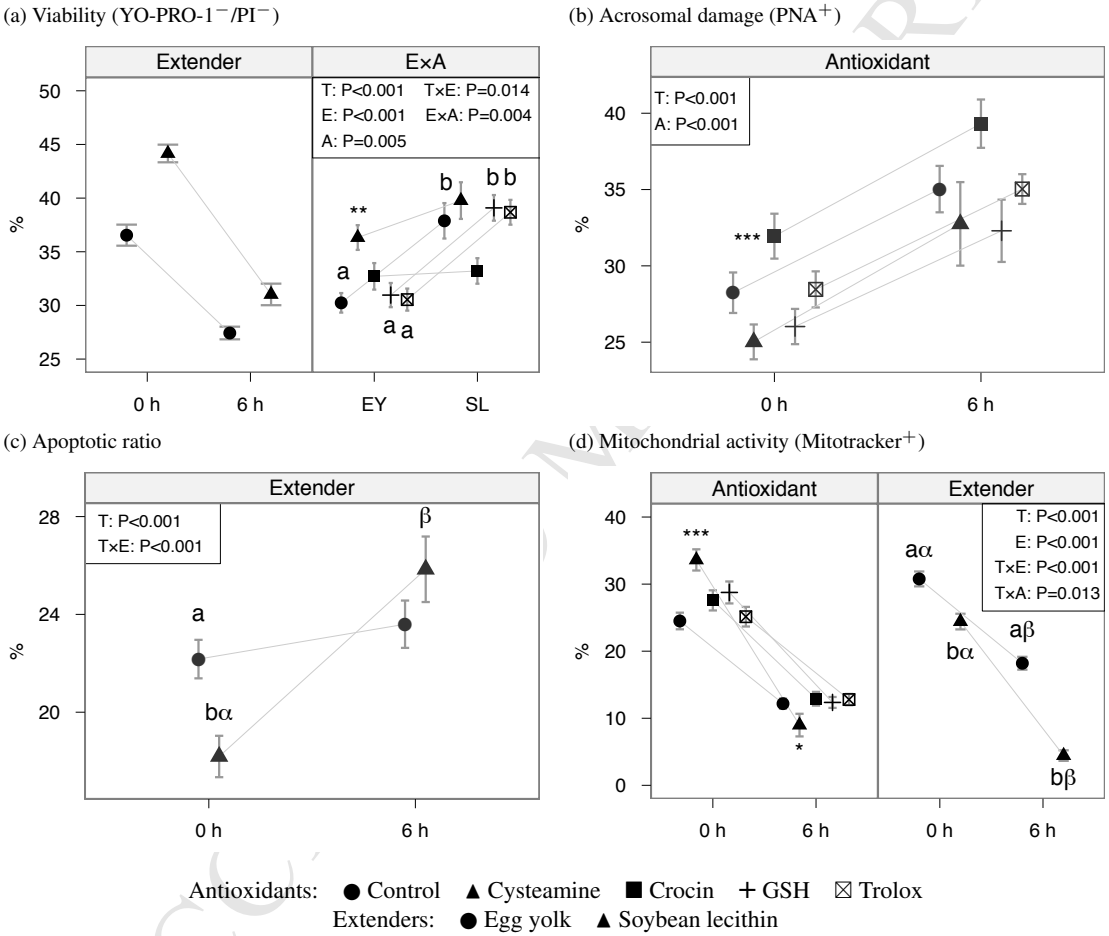
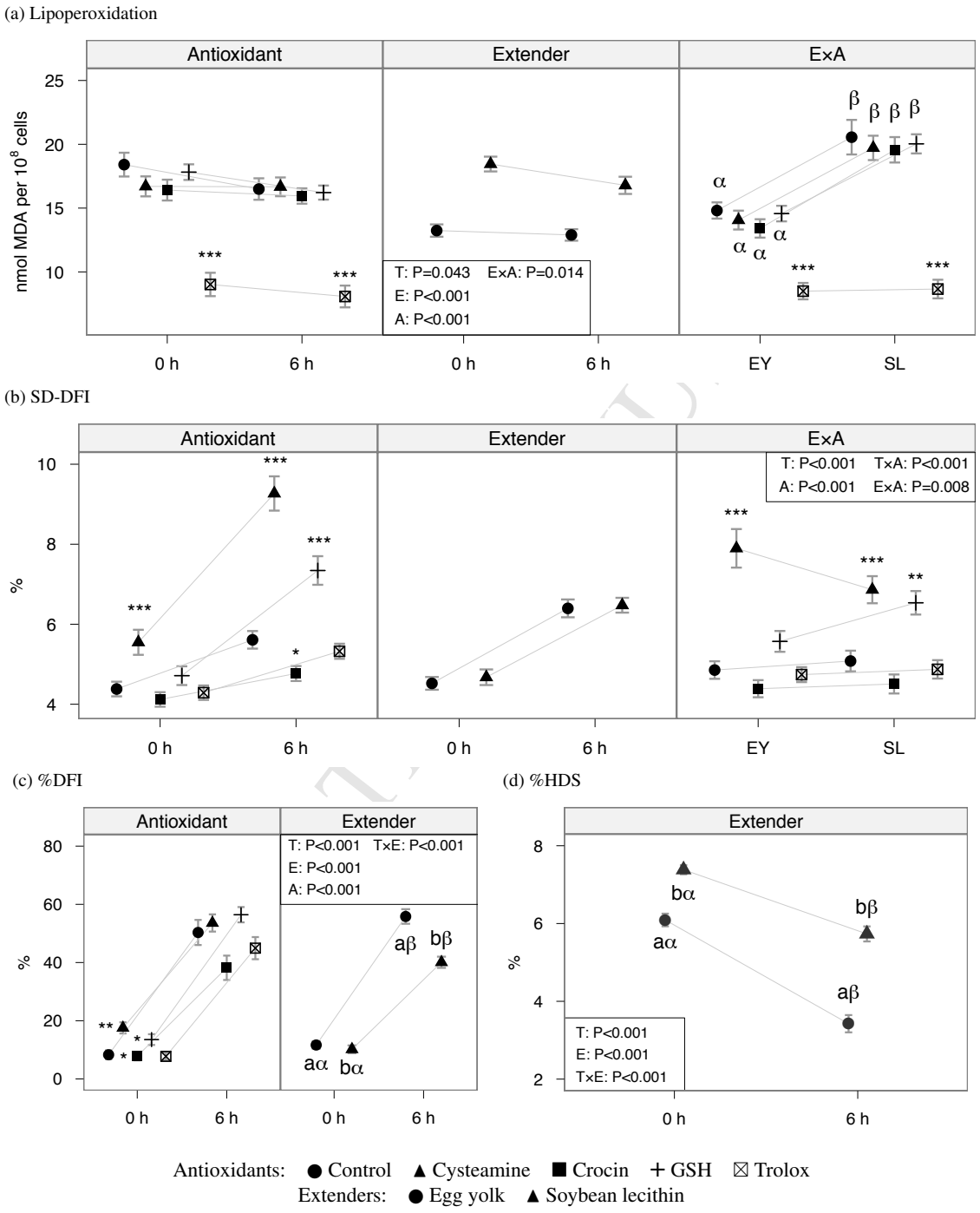

(a) Viability (YO-PRO-1⁻/PI⁻)
Extender
E×A
T: P<0.001 T×E: P=0.014
E: P<0.001 E×A: P=0.004
A: P=0.005
%
0 h
6 h
EY
SL
(b) Acrosomal damage (PNA⁺)
Antioxidant
T: P<0.001
A: P<0.001
(c) Apoptotic ratio
Extender
T: P<0.001
T×E: P<0.001
(d) Mitochondrial activity (Mitotracker⁺)
Antioxidant
Extender
T: P<0.001
E: P<0.001
T×E: P<0.001
T×A: P=0.013
Antioxidants: ● Control ▲ Cysteamine ■ Crocin + GSH ☒ Trolox
Extenders: ● Egg yolk ▲ Soybean lecithin

FIGURE 3


(a) Lipoperoxidation
Antioxidant
Extender
E×A
nmol MDA per 10^8 cells
T: P=0.043 E×A: P=0.014
E: P<0.001
A: P<0.001
0 h
6 h
EY
SL
(b) SD-DFI
%
T: P<0.001 T×A: P<0.001
A: P<0.001 E×A: P=0.008
(c) %DFI
(d) %HDS
T: P<0.001 T×E: P<0.001
E: P<0.001
A: P<0.001
T: P<0.001
E: P<0.001
T×E: P<0.001
Antioxidants: Control Cysteamine Crocin GSH Trolox
Extenders: Egg yolk Soybean lecithin

Highlights

- We have tested two extenders and four antioxidants fror the cryopreservation of ram semen.
- We have tested sperm quality by CASA and flow cytometry post-thawing and after incubating 6 h at 38 °C.
- The effect of the antioxidants is modulated by the extender choice.
- Soy lecithin yields good results but depresses mitochondrial activity.
- Crocin and Trolox seem promising for improving ram semen cryopreservation.