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Cysteine supplementation pre-freeze and post-thaw improves integrity and reduces oxidative stress in cryopreserved ram spermatozoa

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ABSTRACT

Cryopreserved ram sperm is highly sensitive to oxidative stress by reactive oxygen species (ROS) which impair sperm function and integrity. Antioxidants such as cysteine can mitigate the effect of ROS, although the optimal concentration or timing of supplementation is unknown. This study aimed to determine the effect of concentration and timing of cysteine supplementation on the integrity and function of cryopreserved ram spermatozoa. Nine ejaculates were collected from three Texel rams then cryopreserved and supplemented with cysteine (0, 0.5, or 1.0 mg/mL) added pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT) generating seven treatments: 1) control 0 mg/mL, 2) PF 0.5 mg/mL, 3) PF 1 mg/mL, 4) PT 0.5 mg/mL, 5), PT 1.0 mg/mL, 6) PF + PT 0.5 mg/mL and 7) PF + PT 1.0 mg/mL. Sperm motility, viability, acrosome integrity, ROS production and penetrability through artificial cervical mucus were assessed post-thaw. Cysteine supplementation reduced ROS production which thereby improved spermatozoa motility, viability, acrosome integrity and penetrability ($p < 0.001$) Sperm integrity for all parameters was greatest in spermatozoa treated PF + PT with 1.0 mg/mL cysteine, although treatment pre-freeze or post-thaw also improved integrity beyond the control. This study has identified that 1.0 mg/mL cysteine is most beneficial and has highlighted the importance of preventing oxidative stress in spermatozoa post-thaw. These finding can help to mitigate the detrimental effect of cryopreservation on spermatozoa and aid the development of cryopreservation protocols in sheep.

1. Introduction

Within the sheep breeding industry assisted reproductive technologies (ARTs) including artificial insemination (AI) have the potential to maximise the use of genetically superior sires, facilitate breeding of offspring genetically resilient to disease, contain contagious diseases within flocks, and increase productivity sustainably. These advantages are further facilitated with the use of frozen-thawed (FT) semen, enabling long-term storage and ease of transport of gametes. Despite this, the use of cervical AI with FT semen in the sheep breeding industry is limited by pregnancy rates of less than 30 % [15] due to structural damage and impaired function of cryopreserved sperm [27] which reduce the ability of FT sperm to traverse the cervix [12].

Cryopreservation causes the production of reactive oxygen species (ROS) [3] and a reduction in the enzymatic activity of seminal plasma antioxidants [7]. ROS act upon polyunsaturated fatty acids (PUFAs) in the sperm plasma membrane causing extensive damage including lipid peroxidation, oxidation of protein, fragmentation of DNA, and reduced mitochondrial membrane integrity [2]. Antioxidants can reduce or prevent oxidative stress by scavenging ROS [5] and the inclusion of antioxidants in cryopreservation extenders to conserve sperm function has shown promise in numerous species including ram [33], boar [26] and bull [28].

Cysteine is a non-enzymatic amino acid antioxidant of low molecular weight (121 Da). Cysteine can reduce oxidative stress through thiol groups that penetrate the sperm cell membrane enhancing the biosynthesis of glutathione (GSH) a major component of the antioxidant system of spermatozoa which is able to react directly with ROS [7]. Furthermore, cysteine is able to protect sperm membrane lipids and proteins through its direct scavenging action on free radicals [21]. The addition of 5 mM cysteine to ram cryopreservation media improved sperm motility post-thaw [7], and in liquid-stored ram semen 2 mM and 4 mM cysteine increased longevity of spermatozoa through enhanced motility, viability, acrosome integrity and mitochondrial activity [17]. The use of cysteine as a supplementary antioxidant has also shown promise in other species where in liquid-stored boar semen cysteine improves motility,

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membrane integrity, acrosome integrity, and mitochondrial membrane potential and reduces ROS [34] and cysteine supplementation prior to freezing improves acrosome integrity, viability, morphology and motility of freeze-thawed bull sperm [18].

Through its multi-factorial mechanism of action, cysteine may have the potential to mitigate the impact of ROS and thereby enhance the function and integrity of cryopreserved ram spermatozoa. Whilst previous studies have shown promise, a defined protocol for the use of cysteine during cryopreservation and the effect of cysteine on the integrity of cryopreserved ram sperm has not been thoroughly investigated. Specifically, to date there are no published studies that have investigated the effect of concentration and timing of cysteine supplementation on ram sperm integrity, including the ability of sperm to penetrate cervical mucus. Identifying the optimal use of cysteine within ram spermatozoa cryopreservation protocols could advance the development of ram semen storage improving sperm integrity for downstream applications.

This study investigates the effect of cysteine supplementation at different concentrations (0.0, 0.5 and 1.0 mg/mL) pre-freeze and post-thaw on sperm motility, acrosome integrity, viability, ROS production and penetrability through cervical mucus in order to determine the optimal concentration and timing of cysteine supplementation to reduce oxidative stress and maintain sperm function and integrity.

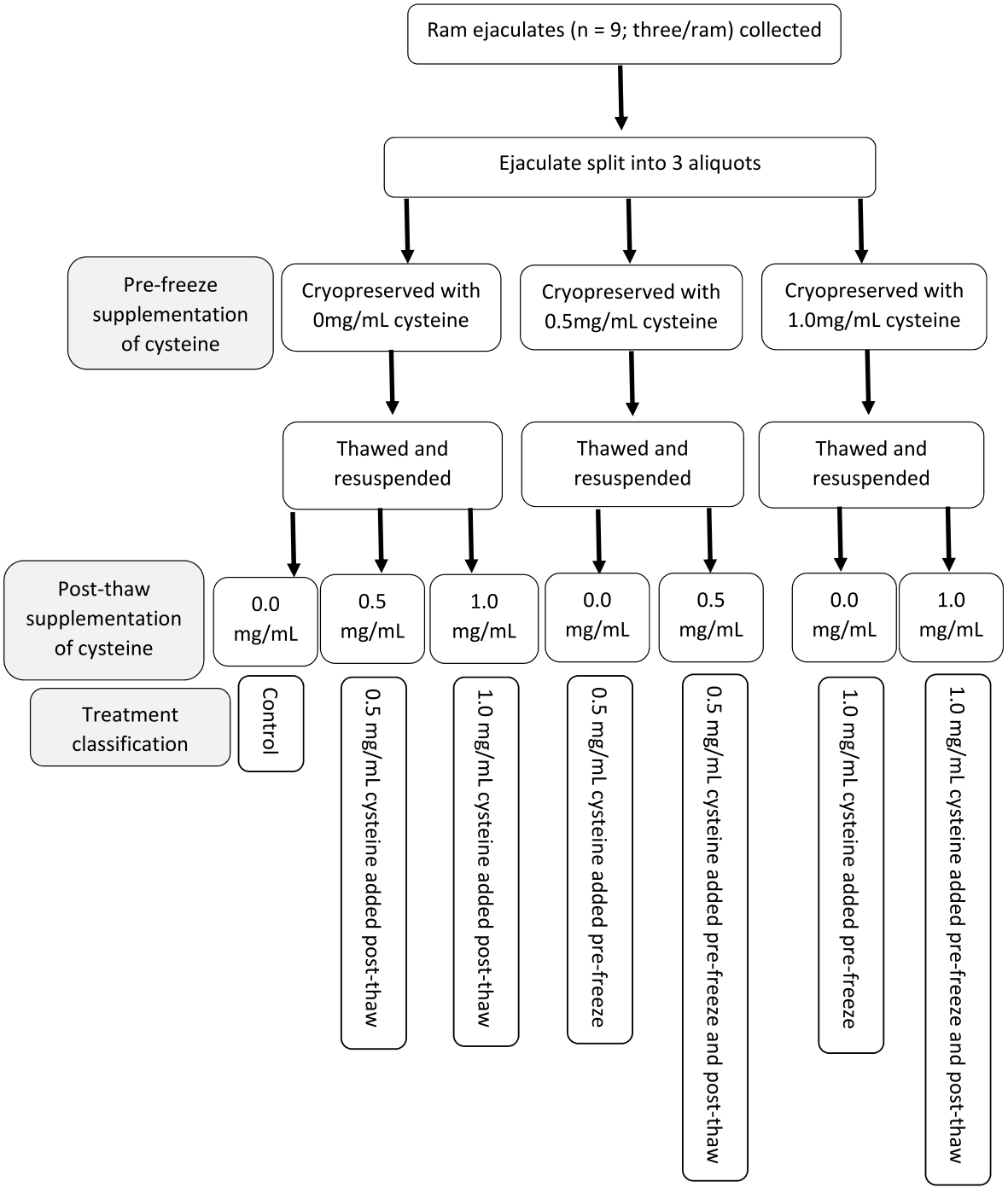

Fig. 1. Diagrammatic representation of experimental protocol illustrating pre-freeze and post-thaw supplementation of ram semen with cysteine at 0.0, 0.5 or 1.0 mg/mL generating seven treatments.

2. Materials and methods

2.1. Animals

Procedures herein were approved by the Harper Adams University ethics committee. Three mature Texel rams with proven fertility aged two to three years were housed indoors at Harper Adams University and fed 1 kg of concentrate daily with hay and water supplied ad libitum.

2.2. Experimental design

Ram ejaculates (n = nine; three/ram) were collected, split into three aliquots and cryopreserved in 0, 0.5 or 1.0 mg/mL cysteine. Next samples were thawed, centrifuged and the sperm pellet resuspended in 0, 0.5 or 1.0 mg/mL cysteine (Fig. 1). This generated seven treatments: 1) control 0 mg/mL, 2) pre-freeze 0.5 mg/mL, 3) pre-freeze 1.0 mg/mL, 4) post-thaw 0.5 mg/mL, 5) post-thaw 1.0 mg/mL, 6) pre-freeze and post-thaw 0.5 mg/mL, and 7) pre-freeze and post-thaw 1.0 mg/mL.

Following thawing and resuspension sperm were held in a water bath at 30 °C and assessed for motility, viability, and acrosome integrity at 0, 30, 60 and 180 min after resuspension; ROS at 0, 60 and 180 min, and the penetrability assay was started 0min following resuspension.

2.3. Semen cryopreservation and thawing

Nine ejaculates were collected from three trained rams (three ejaculates per ram) via artificial vagina (IMV technologies, L'Aigle, France) during the breeding season (November). Immediately after collection, the ejaculates were immersed in a water bath at 35 °C. Within 3 min of collection semen samples were assessed for volume, concentration and motility as described below. Only ejaculates with volume 1–2 mL, sperm concentration $>3 \times 10^9$ sperm/mL and progressive motility >80 % were used in the study.

Next, ejaculates were split into three aliquots and cryopreserved in tris-citrate-glucose cryodiluent (TCG) (final concentrations: 300 mM Tris, 94.7 mM citric acid, 27.8 mM glucose, 12 % egg yolk, 4 % glycerol) supplemented with cysteine at 0, 0.5 or 1.0 mg/mL. Ejaculates were diluted dropwise 1:4 (semen:cryodiluent v:v) in warm (35 °C) TCG containing cysteine then chilled to 4 °C over 2 h in a water jacket. The chilled samples were frozen as 200 μL pellets on dry ice for 2 min then plunged into liquid nitrogen and stored as described previously [14] until post-thaw treatment and analysis.

L-Cysteine (121.16 g/mol; Sigma-Aldrich, UK) was diluted to a stock solution of 100 mg/mL in 0.02 M Dulbeccos PBS and added to the TCG at a concentration of 1.25 mg/mL to give a working concentration of 1.0 mg/mL (8.25 mM) when diluted 1:4 semen:diluent and 0.625 mg/mL to give a working concentration of 0.5 mg/mL (0.41 mM) when diluted 1:4 semen:cryodiluent. A third treatment contained no cysteine and acted as a control (0 mg/mL) generating three pre-freeze cysteine concentrations of 0, 0.5 and 1.0 mg/mL.

Cryopreserved samples were thawed in a water bath in a clean dry glass test-tube with vigorous shaking for 2 min at 37 °C. Thawed samples were centrifuged at 300×*g* for 3 min, the sperm pellet resuspended in TCG and centrifuged at 300×*g* for 3 min then the pellet resuspended in TCG containing 0, 0.5 or 1.0 mg/mL cysteine to a final spermatozoa concentration of 50×10^6 sperm/mL. The TCG washing step was incorporated to remove any residual cysteine within the sample to ensure post thaw concentrations of cysteine were reliable. A post-thaw concentration of 50×10^6 sperm/mL was used as this is the optimal concentration for the assessments used within the study and as such prevented any further processing of the spermatozoa post-thaw for individual assessments.

2.4. Assessment of ram spermatozoa

2.4.1. Proportion of motile spermatozoa

Progressive motility was assessed to the nearest 5 % on a scale of 0–100 % at 0, 30, 60 and 180 min after thawing and resuspension (hereafter referred to as post-thaw). 10 μL of semen was placed on a pre-warmed (37 °C) slide and covered with a warm 22 × 22 mm coverslip then assessed at ×100 magnification using phase contrast microscopy (Olympus CX31, Tokyo, Japan).

2.4.2. Acrosome integrity and viability

Acrosome integrity and viability were assessed at 0, 30, 60 and 180 min post-thaw using fluorescein-conjugated peanut agglutinin (FITC-PNA; Sigma-Aldrich, USA) and propidium iodide (PI; Sigma-Aldrich) based on a previously published protocol [6]. Briefly, aliquots of 40 μL of spermatozoa (50×10^6/mL) were incubated with 10 μL of FITC-PNA (working concentration 40 μg/mL) and 0.5 μL of 2.4 mM PI for 5 min at 37 °C. Sperm samples were then fixed in 0.1 % formaldehyde (Sigma-Aldrich, UK) at room temperature for 5 min. Next 20 μL was placed on a glass slide under a cover slip (24 × 50 mm) and 200 cells per sample were assessed at ×40 magnification using fluorescent microscopy (excitation filter 460–495 nm, emission filter 510–560 nm excitation filter 530–560 nm emission filter 570–640 nm, Leica LEITZ DMRB, Leica Microsystems, Germany).

2.4.3. Sperm penetration test

Sperm penetration was assessed immediately post-thaw only. Artificial cervical mucus was prepared as described previously [24] containing 1.6 % acrylamide gel, 0.03 % ammonium persulfate and 0.05 % TEMED. Preliminary tests (n = 5 ejaculates, n = 2 repetitions per ejaculate) in our laboratory indicated that there was no significant difference (mean ± sem) in sperm penetrability between natural cervical mucus collected at oestrus (15.4 mm/h) and the artificial cervical mucus used in this study (17.8 mm/h; $p = 0.28$). The intra-assay CV for natural and artificial cervical mucus was 18.7 % and 10.5 % respectively. It is therefore expected that this assay would generate results indicative of those if using cervical mucus collected from ewes at oestrus. Micro haematocrit tubes (0.3 × 0.3 × 7.5 mm; Hawksley, London, UK) were loaded with artificial mucus (37 °C) using capillary action then the tube was sealed at one end with Crista Seal (Hawksley, London, UK). Next the capillary tube was positioned vertically and placed in a 0.5 mL microcentrifuge tube containing 200 μL of the sperm sample (50×10^6/mL) and incubated at 37 °C for 1 h. Following incubation, the capillary tube was sealed at the open end and incubated at −20 °C for 2 min to halt sperm motility. The tube was examined under light microscopy at ×40 magnification and the distance reached by the furthest spermatozoon (Vanguard distance) was recorded to the nearest mm.

2.4.4. ROS detection by nitroblue tetrazolium (NBT)

The presence of ROS was observed at 0, 60 and 180 min post-thaw using 0.1 % nitroblue tetrazolium (NBT) which causes the formation of formazan in the presence of ROS as previously described for spermatozoa and leukocytes [13]. Equal volumes (100 μL) of 0.1 % NBT solution and thawed semen (see section 2.3) at 50×10^6 sperm/mL were incubated at 37 °C for 30 min. Next samples were centrifuged at 250×*g* for 5 min, the supernatant discarded and the pellet resuspended in 200 μL PBS. Sperm smears (20 μL) were prepared on glass slides, air dried at room temperature for 5 min and immersed in Wright stain (0.3 %; Sigma, Poole UK) for 2 min at room temperature. Smears were washed in tap water for 3–4 min until the desired level of pink staining was achieved and air dried at room temperature. A total of 200 spermatozoa were scored at ×100 magnification using light microscopy. The proportion of the sperm head that stained positive for formazan was assessed as 0 %, <50 %, >50 % or 100 % as used previously with high proportions of staining indicative of oxidative stress [13].

The NBT ROS assay shows strong correlation with the commonly

used luminol chemiluminescence assay for assessment of ROS production [13]. In human spermatozoa, the production of ROS when assessed by the NBT assay was positively correlated with sperm DNA fragmentation and negatively correlated with sperm motility [32] and higher ROS concentrations as determine by NBT staining were associated with infertility [4]. Commercially available assays for ROS assessment using the NBT test assess sperm staining as high, medium or low, similarly to the assessment used in the present study, with "high" staining associated with infertility [8]. It has been suggested that the NBT staining assay is less specific than the chemiluminescence assay as seminal plasma contains reductases which can reduce the NBT stain to formazan in the absence of ROS [8], and therefore in the present study ROS was assessed in spermatozoa in the absence of seminal plasma following centrifugation and resuspension.

2.5. Statistical analyses

Penetrability of spermatozoa through cervical mucus was analyzed by one way ANOVA blocked for ram whereby penetrability was included as the Y-variate and cysteine supplementation was included as treatment. All other sperm assessments were analyzed using a repeated measures ANOVA with a linear mixed model (REML) in Genstat version 19 (VSN International, Hemel Hempstead UK). Treatment and time and their interaction were included in the fixed model, and when there was no interaction this was removed from the fixed model. To accommodate the experimental design, the random effects were treatment nested with ejaculate nested within ram. For all data $P < 0.05$ was considered statistically significant and post-hoc analyses were performed using the least significant difference (LSD) test.

3. Results

3.1. The effect of cysteine supplementation on the proportion of progressively motile spermatozoa

There was an interaction between treatment and time ($P < 0.001$; Table 1) on progressive motility. Motility was generally greatest at all time points in spermatozoa treated with 1.0 mg/mL cysteine both pre-freeze and post-thaw, intermediate when treated with 0.5 mg/mL both pre-freeze and post-thaw, and lowest in spermatozoa treated only pre-freeze or post-thaw. At 30, 60 and 180 min post-thaw the control had significantly lower motility than all other treatments. In spermatozoa treated with 1.0 mg/mL cysteine both pre-freeze and post-thaw motility at 180 min was 24 %. Motility decreased significantly over time from 0min to 180min post-thaw in all treatments (Table 1).

3.2. The effect of cysteine supplementation on the percentage of viable spermatozoa

There was an interaction between treatment and time ($P < 0.001$; Table 2) on the proportion of viable spermatozoa. Viability tended to be highest in spermatozoa treated with 1.0 mg/mL or 0.5 mg/mL cysteine both pre-freeze and post-thaw and intermediate in spermatozoa treated with cysteine only pre-freeze or post-thaw although this varied between time points (Table 2).

At all time points viability was significantly lower in the control (no cysteine) than all other treatments. The proportion of viable sperm decreased significantly over time from 0 min to 180 min post-thaw in all treatment groups.

3.3. The effect of cysteine supplementation on the percentage of spermatozoa with intact acrosomes

There was no interaction between treatment and time ($P = 0.069$) although there was an effect of treatment ($P < 0.001$) and time ($P < 0.001$). Acrosome integrity (mean ± SEM) was greatest in spermatozoa treated with 1.0 mg/mL or 0.5 mg/mL cysteine both pre-freeze and post thaw, lowest in the control (Table 3).

Acrosome integrity (mean ± SEM) decreased over time being greatest at 0 min (55.54 ± 0.636), then declining at 30 min (50.68 ± 0.748) 60 min (45.08 ± 0.827), and 180 min (26.81 ± 0.697), with each time point being significantly different.

3.4. The effect of cysteine supplementation on the penetrability of spermatozoa through artificial cervical mucus

There was an effect ($P < 0.001$) of treatment on the distance travelled through artificial cervical mucus by spermatozoa (Fig. 2). Spermatozoa treated with 1.0 mg/mL cysteine both pre-freeze and post-thaw travelled further than all other treatments, followed by 0.5 mg/mL pre-freeze and post-thaw and 1.0 mg/mL post-thaw which were not significantly different from each other. Distance travelled was significantly lower in the control than all other treatments.

3.5. The effect of cysteine supplementation on the proportion of spermatozoa with ROS at the sperm head

The proportion of spermatozoa with 0 %, <50 %, >50 % or 100 %

Table 1
Mean (±SEM) % motility of frozen-thawed ram spermatozoa at 0, 30, 60, and 180 min post-thaw when supplemented pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT) with 0 (control) 0.5 or 1.0 mg/mL cysteine.

Treatment	0 min	30 min	60 min	180 min
Control	50.00 ± 0.00^{aw}	29.44 ± 1.002^{ax}	15.78 ± 1.140^{ay}	0.56 ± 0.556^{az}
PF 0.5 mg/mL	50.67 ± 0.333^{aw}	40.44 ± 0.884^{bcx}	31.89 ± 0.992^{by}	10.78 ± 0.662^{bz}
PF 1.0 mg/mL	51.67 ± 0.726^{abw}	43.89 ± 0.790^{cdx}	34.89 ± 1.241^{bcy}	16.44 ± 1.396^{cz}
PT 0.5 mg/mL	50.22 ± 0.222^{aw}	40.11 ± 0.676^{bx}	32.22 ± 1.211^{by}	10.78 ± 1.310^{bz}
PT 1.0 mg/mL	52.11 ± 0.772^{abw}	42.89 ± 0.841^{bcdx}	35.00 ± 1.312^{bcy}	16.78 ± 0.741^{cz}
PF + PT 0.5 mg/mL	54.78 ± 0.222^{bcw}	46.22 ± 0.760^{dx}	38.44 ± 1.625^{cy}	18.00 ± 0.866^{cz}
PF + PT 1.0 mg/mL	58.00 ± 0.645^{cw}	50.56 ± 0.7290^{ex}	43.89 ± 0.935^{dy}	24.00 ± 0.553^{dz}

Within columns, means with different superscripts $^{a\text{-}d}$ differ significantly ($p < 0.05$).
Within rows, means with different superscripts $^{w\text{-}z}$ differ significantly ($p < 0.05$).

Table 2
Mean (±SEM) % viability of frozen-thawed ram spermatozoa at 0, 30, 60, and 180 min post-thaw when supplemented pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT) with 0 (control) 0.5 or 1.0 mg/mL cysteine.

Treatment	0 min	30 min	60 min	180 min
Control	50.67 ± 0.577^{aw}	43.89 ± 0.655^{ax}	36.00 ± 0.667^{ay}	22.11 ± 0.716^{az}
PF 0.5 mg/mL	55.44 ± 0.242^{bcw}	50.33 ± 0.645^{bx}	44.44 ± 0.988^{by}	26.78 ± 0.760^{bz}
PF 1.0 mg/mL	57.22 ± 0.434^{bcdw}	51.33 ± 1.047^{bx}	46.22 ± 1.362^{by}	26.78 ± 0.778^{bz}
PT 0.5 mg/mL	56.11 ± 0.351^{bcw}	51.89 ± 0.564^{bx}	45.44 ± 0.868^{by}	28.11 ± 0.889^{bz}
PT 1.0 mg/mL	57.56 ± 0.648^{bcdw}	52.89 ± 1.207^{bcx}	46.89 ± 1.620^{bcy}	27.67 ± 0.882^{bz}
PF + PT 0.5 mg/mL	59.44 ± 0.503^{bdw}	56.00 ± 0.373^{cdx}	50.33 ± 0.645^{cdy}	35.33 ± 0.928^{cz}
PF + PT 1.0 mg/mL	60.11 ± 0.807^{dw}	56.44 ± 0.626^{dx}	51.56 ± 0.852^{dy}	35.56 ± 1.642^{cz}

Within columns, means with different superscripts $^{a\text{-}d}$ differ significantly ($p < 0.05$).
Within rows, means with different superscripts $^{w\text{-}z}$ differ significantly ($p < 0.05$).

Table 3
Mean (±SEM) % acrosome integrity of frozen-thawed ram spermatozoa when supplemented pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT) with 0 (control), 0.5, or 1.0 mg/mL cysteine.

Treatment	Acrosome Integrity
Control	37.41 ± 01.935[a]
PF 0.5 mg/mL	41.14 ± 1.862[b]
PF 1.0 mg/mL	42.83 ± 2.116[b]
PT 0.5 mg/mL	44.25 ± 1.932[c]
PT 1.0 mg/mL	46.31 ± 2.022[d]
PF + PT 0.5 mg/mL	48.28 ± 1.796[d]
PF + PT 1.0 mg/mL	51.47 ± 1.939[e]

Means with different superscripts [a-e] differ significantly ($p < 0.05$).

ROS production at the sperm head were analyzed independently. For all data sets there was an interaction between treatment and time ($P < 0.001$; Table 4). The proportion of spermatozoa with 0 or <50 % ROS at the sperm head decreased from 0 min to 180 min post-thaw with each time point being different ($P < 0.05$). Conversely spermatozoa with >50 or 100 % ROS at the sperm head increased from 0 min to 180 min post-thaw with each time point being different ($P < 0.05$).

The proportion of spermatozoa with 0 % and 50 % ROS tended to be higher in samples treated both pre-freeze and post-thaw with 0.5 or 1.0 mg/mL cysteine compared to other treatments and lowest in the control. Conversely the proportion of spermatozoa with >50 % or 100 % ROS at the sperm head tended to be lower in samples treated pre-freeze and post-thaw with 0.5 or 1.0 mg/mL cysteine compared to other treatments. There tended to be little difference in the proportion of spermatozoa with 0, <50, >50 or 100 % ROS at the sperm head when treated either pre-freeze or post-thaw with 0.5 mg/mL and 1.0 mg/mL cysteine (Table 4).

4. Discussion

In the present study, supplementation of cryopreserved ram ejaculates with cysteine improved spermatozoa motility, viability, acrosome integrity and penetration through cervical mucus, whilst also reducing the proportion of spermatozoa with >50 or 100 % ROS at the sperm head.

During cryopreservation temperature changes generate reactive oxygen species (ROS) inducing oxidative stress which has two predominant effects: destabilsation of the DNA backbone leading to DNA damage and subsequent cell death [30] and lipid peroxidation of sperm membrane PUFAs which causes loss of biological function [2] that can be observed via a decline in sperm motility, viability and acrosome integrity [5].

Within the present study cysteine decreased ROS as determined using Nitro-blue tetrazolium which reacts with cellular superoxide anions. This reaction generates formazan which is visualized as blue staining [13]. Cysteine directly scavenges free radicals including superoxide anion (O_2-) and hydrogen peroxide (H_2O_2) [21] and is proposed to mediate intracellular catalase and glutathione (GSH) antioxidant activity. Despite suggestions that mammalian spermatozoa contain limited catalase [2], cysteine supplementation increases the concentration of catalase in frozen-thawed ram semen [7,9]. Furthermore, cysteine can permeate the sperm cell membrane enhancing the biosynthesis of GSH which scavenges free radicals and is a precursor to glutathione peroxidase (GPx) an enzymatic antioxidant [5] although cysteine does not increase GPx [7,9] or GSH [7] concentrations in cryopreserved ram semen. Consequently, in the present study it is likely that reduced ROS is predominantly due to direct scavenging of superoxide anions, although it is also possible that the antioxidants catalase and GSH are activated by cysteine to protect spermatozoa from ROS.

ROS production was lowest when spermatozoa were supplemented with cysteine both before and after cryopreservation. The beneficial effect of supplementing ram spermatozoa with cysteine post-thaw has

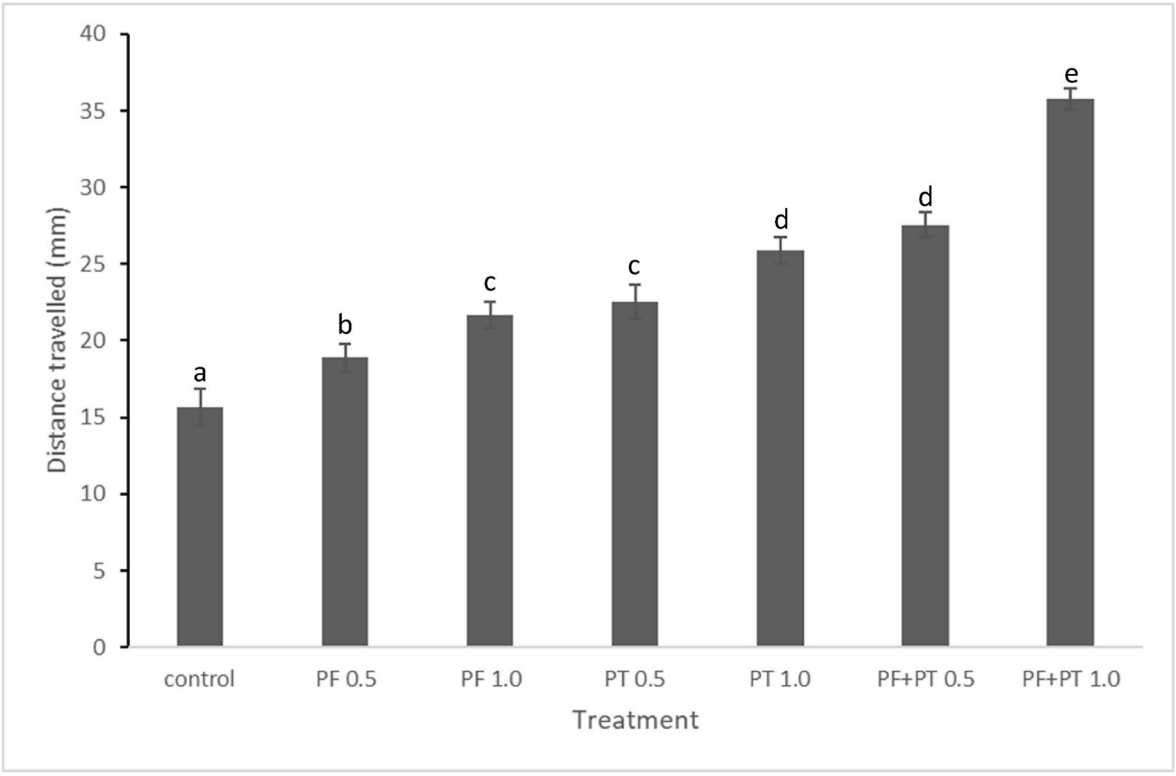

Fig. 2. Mean (±SEM) distance travelled (mm) by ram spermatozoa through artificial cervical mucus when supplemented pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT) with 0 (control) 0.5 or 1.0 mg/mL cysteine. Means without a common superscript [a-e] differ significantly ($p < 0.001$).

Table 4
Mean (% ± SEM) proportion of frozen-thawed ram spermatozoa with 0, <50, >50 or 100 % ROS staining at the sperm head at 0, 30, 60 and 180 min post-thaw when supplemented pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT) with 0 (control) 0.5 or 1.0 mg/mL cysteine.

% ROS staining	Treatment	0 min	30 min	60 min	180 min
0 %	Control	10.33 ± 1.027^{a}	6.22 ± 0.954^{a}	1.56 ± 0.530^{a}	0.00 ± 0^{a}
	PF 0.5 mg/mL	16.67 ± 0.5^{b}	11.44 ± 0.648^{b}	6.67 ± 0.471^{b}	0.00 ± 0^{a}
	PF 1.0 mg/mL	19.44 ± 0.818^{b}	13.78 ± 0.687^{b}	6.89 ± 0.807^{b}	0.33 ± 0.033^{a}
	PT 0.5 mg/mL	18.22 ± 0.954^{b}	12.67 ± 0.726^{b}	5.89 ± 0.790^{b}	0.0 ± 0^{a}
	PT 1.0 mg/mL	19.44 ± 0.729^{b}	13.56 ± 0.972^{b}	6.89 ± 0.807^{b}	0.0 ± 0^{a}
	PF + PT 0.5 mg/mL	25.78 ± 0.521^{c}	19.78 ± 0.547^{c}	12.11 ± 0.611^{c}	4.56 ± 0.475^{b}
	PF + PT 1.0 mg/mL	28.78 ± 0.745^{d}	23.11 ± 0.889^{d}	15.00 ± 0.928^{c}	5.78 ± 0.619^{b}
<50	Control	42.78 ± 1.245^{ab}	36.11 ± 1.207^{bc}	26.44 ± 1.591^{bc}	3.78 ± 0.619^{c}
	PF 0.5 mg/mL	35.11 ± 1.495^{c}	30.56 ± 2.115^{c}	24.89 ± 2.118^{bc}	6.78 ± 0.778BCE
	PF 1.0 mg/mL	40.33 ± 1.143^{abc}	34.56 ± 1.292^{bc}	28.89 ± 1.550b^{c}	7.56 ± 0.709^{b}
	PT 0.5 mg/mL	39.67 ± 1.77^{abc}	33.11 ± 1.822^{ab}	24.33 ± 1.491^{c}	6.67 ± 0.527^{b}
	PT 1.0 mg/mL	37.78 ± 0.846^{bc}	32.56 ± 1.056^{ab}	26.11 ± 1.662^{ab}	8.11 ± 0.735^{b}
	PF + PT 0.5 mg/mL	43.78 ± 1.164^{a}	38.89 ± 1.317^{a}	33.11 ± 1.419^{a}	15.56 ± 0.818^{a}
	PF + PT 1.0 mg/mL	41.00 ± 0.726^{ab}	36.89 ± 1.184^{a}	31.67 ± 1.572^{a}	15.11 ± 1.184^{a}
>50	Control	20.67 ± 0.882^{b}	24.56 ± 0.801^{b}	28.67 ± 1.0.54^{a}	35.22 ± 0.813^{a}
	PF 0.5 mg/mL	24.22 ± 1.064^{c}	29.54 ± 1.492^{c}	34.89 ± 1.338^{b}	47.00 ± 0.957^{c}
	PF 1.0 mg/mL	20.56 ± 0.835b^{c}	26.33 ± 0.726^{b}	33.56 ± 1.226^{b}	49.56 ± 0.603^{c}
	PT 0.5 mg/mL	21.44 ± 1.015b^{c}	26.67 ± 1.130^{bc}	34.22 ± 1.451^{b}	47.44 ± 1.365^{bc}
	PT 1.0 mg/mL	22.44 ± 0.709^{bc}	28.44 ± 0.853^{bc}	35.33 ± 1.213^{b}	49.11 ± 0.588^{c}
	PF + PT 0.5 mg/mL	15.11 ± 0.949^{a}	21.00 ± 1.130^{a}	28.89 ± 1.695^{a}	45.78 ± 0.969^{b}
	PF + PT 1.0 mg/mL	16.89 ± 0.512^{a}	22.44 ± 1.180^{a}	29.11 ± 1.940^{a}	45.89 ± 1.073^{b}
100	Control	26.22 ± 0.846^{e}	33.11 ± 0.889^{c}	43.33 ± 1.333^{d}	61.00 ± 0.957^{d}
	PF 0.5 mg/mL	24.00 ± 0.764^{de}	28.56 ± 0.899^{b}	33.33 ± 1.258^{bc}	46.22 ± 0.572^{c}
	PF 1.0 mg/mL	19.67 ± 1.080^{ab}	25.33 ± 1.027^{b}	30.67 ± 1.247^{b}	42.56 ± 0.747^{b}
	PT 0.5 mg/mL	20.67 ± 0.866^{cd}	27.56 ± 0.729^{b}	35.56 ± 1.556^{c}	45.89 ± 978^{bc}
	PT 1.0 mg/mL	20.33 ± 0.816^{cd}	25.44 ± 1.056^{b}	31.67 ± 1.213b^{c}	42.78 ± 0.862^{bc}
	PF + PT 0.5 mg/mL	15.33 ± 0.441^{ab}	20.33 ± 0.687^{a}	25.89 ± 0.539^{a}	34.11 ± 1.073^{a}
	PF + PT 1.0 mg/mL	13.33 ± 0.645^{a}	17.56 ± 0.709^{a}	24.22 ± 1.234^{a}	33.22 ± 1.470^{a}

Within columns for each % ROS staining (0, <50, >50 and 100 %), means with different superscripts $^{a\text{-}e}$ differ significantly ($p < 0.05$).

not previously been described, and this finding suggests a requirement, and ability, to prevent oxidative stress and subsequent damage in sperm cells following thawing. Reactive oxygen species cause lipid peroxidation (LPO) of PUFAs within the sperm membrane creating a cascade whereby LPO causes further ROS generation by sperm mitochondria resulting in a self-propagating cycle leading to cell death [1]. Cryopreservation of spermatozoa induces oxidative stress thus increasing LPO and resulting in sperm populations post-thaw that generate ROS. It is proposed that in the present study, the addition of cysteine post-thaw helped to reduce the negative impact of ROS from impaired spermatozoa thus slowing the LPO cascade effect after thawing. Interestingly, the addition of cysteine either pre-freeze or post-thaw had similar effects to the level of ROS at the sperm head suggesting that ROS generated during cryopreservation can be mitigated post-thaw. This could be advantageous in the industry enabling supplementation of semen that is already cryopreserved to enhance sperm integrity.

Cysteine supplementation both pre-freeze and post-thaw improved sperm viability by more than 10 % compared to control most likely by protecting the sperm membrane from free radicals. In agreement with the present study cysteine improved the viability of frozen-thawed [9] and liquid-stored [17] ram spermatozoa at concentrations of 2–4 mM (compared to 8.25 mM in the present study: 1.0 mg/mL) and improved viability of frozen-thawed bull spermatozoa [18].

In previously published studies, cysteine added pre-freeze to ram spermatozoa did not significantly improve acrosome integrity [7,9] but did improve acrosome integrity of bull spermatozoa [29]. Cryopreservation induces acrosomal damage and ram spermatozoa are more susceptible to this than bull [31]. It is possible that in the present study, the higher concentration of cysteine used, as well as the post-thaw addition of cysteine, helped to mitigate the negative impacts of cryopreservation in ram spermatozoa. ROS induces capacitation and the acrosome reaction [20], and it is therefore likely that the reduced ROS in the presence of cysteine conserves acrosome integrity. In the present study acrosome integrity was greatest in samples treated both pre-freeze and post-thaw or those treated post-thaw only implying that acrosomal damage is greatest post-thaw and that cysteine can help to mitigate the impact of thawing.

Cysteine supplementation improved sperm motility, especially at concentrations of 1.0 mg/mL. Similarly 5 mM cysteine increased post-thaw motility of ram spermatozoa [7] whereas 1–4 mM cysteine had no effect [9]. It is possible that higher concentrations of cysteine, such as that used in the present study (8.25 mM) are required to significantly enhance motility. Importantly, in the present study, 1.0 mg/mL cysteine both pre-freeze and post-thaw maintained motility of cryopreserved sperm at 50 % (20 % greater than control samples) at 30min post-thaw, suggesting that post-thaw addition of cysteine improves longevity. Sperm motility is correlated with fertility in numerous species including ram [10], bull [23] and human [11], suggesting cysteine supplementation has potential to enhance sperm function in vivo, however this cannot be confirmed without robust fertility studies. Sperm motility is mediated by the mitochondria-rich axoneme and dense fibers of the mid-piece that utilize intracellular ATP to generate energy [22]. High concentrations of ROS can reduce ATP production causing insufficient phosphorylation of the axoneme and hence loss of motility [20], thus in the present study it is likely that the action of cysteine on ROS and membrane integrity enhanced motility.

Cysteine supplementation pre-freeze and post-thaw increased the penetrability of spermatozoa through artificial cervical mucus. Penetrability of ram spermatozoa in artificial mucus is positively correlated with motility and negatively correlated with the proportion of spermatozoa with damaged acrosomes, although is not correlated with sperm viability [24]. Likewise, penetration of bull spermatozoa in cervical mucus is correlated with acrosome integrity [16]. Consequently, in the present study, the increased penetrability of spermatozoa were likely mediated by the increased motility and acrosome integrity. The effect of treatment showed a similar response in penetrability to acrosome integrity with post-thaw addition of cysteine having the greatest effect in both parameters suggesting that acrosome integrity may be an important factor in sperm penetrability.

The present study has: identified a significant positive effect of cysteine supplementation on the integrity of cryopreserved ram spermatozoa, for the first time determined the effect of cysteine supplementation on the penetrability of sperm through cervical mucus, and to our knowledge is the first study to highlight the benefits of cysteine supplementation post-thaw on sperm integrity. Whilst these results are

positive and can aid the development of semen cryopreservation protocols, further investigation is required to ascertain the impact of cysteine supplementation on sperm fertilizing potential. Antioxidants are reported to improve sperm integrity in a range of species [25], though there are limited reports of their impact on fertility and pregnancy rates. In buffalo, the use of 2 mM cysteine increased pregnancy rates from 43 % (control) to 59 % [19] suggesting a potential benefit. In sheep, the use of cryopreserved semen supplemented with cysteine alongside artificial insemination and other assisted reproductive technologies including in-vitro production of embryos requires investigation.

In conclusion supplementation of cryopreserved ram spermatozoa with 0.5 or 1.0 mg/mL cysteine reduces ROS concentration thereby enhancing motility, viability and acrosome integrity, and subsequently increases the ability of spermatozoa to penetrate cervical mucus. The beneficial effect of using cysteine after thawing has not been previously demonstrated, and this study highlights the importance of protecting spermatozoa from oxidative stress post-thaw as well as during cooling and freezing. Furthermore, the high concentration of cysteine used in this study demonstrated beneficial effects on sperm function. The use of cysteine as an antioxidant has the potential to mitigate the negative effects of cryopreservation and may aid the development of sperm cryopreservation protocols in sheep. There is a need to examine the fertilizing potential of ram sperm supplemented with cysteine to ascertain its potential in aiding the development of assisted reproductive technologies in the sheep breeding industry.

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Declarations of competing interest

None.

CRediT author statement

Ahmed Kafi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. Tharangani Herath: Conceptualization, Supervision. Muhammad Khalid: Conceptualization, Writing – Review and editing, Supervision. Claire Kershaw: Conceptualization, Methodology, Formal analysis, Writing – original draft, Supervision.

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