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USE PUNICA GRANATUM SEED EXTRACT AS A BOTANICAL ANTIOXIDANT FOR ENHANCING COLD-PRESERVED (5°C) SHEEP EPIDIDYMAL SPERMATOZOA QUALITY

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ABSTACT

One of the most significant challenges in reproductive biotechnology is preserving male gametes during hypothermic storage due to oxidative stress-induced cellular injury during storage. The goal of this study was to investigate whether a natural antioxidant such as pomegranate (Punica granatum) seed extract could preserve sheep epididymal spermatozoa at 5 degrees Celsius when added to a Tris-Citrate-Fructose yolk (TCFY) extender. Spermatozoa were collected from freshly processed goat testis and diluted in TCFY extender containing different concentrations of pomegranate juice: 0% (control), 5%, 10%, 15%, and 20%. Sample were analysed at 24, 48, 72, 96, and 120 hours post-storage for sperm progressive motility, sperm plasma membrane integrity (by HOST testing), sperm viability (by eosin-nigrosin staining) and morphological normalcy. In comparison to the controls and higher concentrations of PJ 10% PJ concentrations provided significant preservation of spermatozoa quality parameters. Spermatozoa in the 10% PJ exhibited the highest level of progressive motility (87.39 at 24h) and had the highest level of plasma membrane integrity (84.90) and viability (81.04) and had the highest level of Morphological normality (89.78) . On the other hand, the 20% concentration of PJ demonstrated a deleterious effect on spermatozoa with the highest degree of damage to plasma membrane integrity (17.12 at 120h). Collectively, these findings indicate that moderate JP concentrations (10%) have capabilities of counteracting oxidative damage to spermatozoa produced during cold storage due to their high polyphenolic content, while excess concentrations of PJ result in pro-oxidative damage to spermatozoa. Therefore, from this study, optimal protocols for pomegranate extract provide for improved preservation of sheep epididymal spermatozoa in sheep artificial insemination programs.

Keywords: Pomegranate extract; Epididymal spermatozoa; Cold preservation; Antioxidant; Sheep; Oxidative stress

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Introduction

Modern reproductive biotechnology is heavily reliant on the ability to conserve male gametes via refrigeration and cryopreservation techniques, which allow for genetic enhancement initiatives, artificial insemination, the preservation of endangered species and posthumous sperm retrieval (1). Currently, in the field of veterinary medicine, the storage of spermatozoa increases the length of breeding seasons, facilitates the exchange of international germplasm, and allows for strategic selection of animals with superior genetic lines to enhance livestock productivity (2). Epididymally-collected spermatozoa, stored from the cauda epididymis after an animal has died or been castrated, provides an opportunity to rescue valuable genetic material from males with genetically preferable characteristics who have died unexpectedly (3). However, the process of cooling and preserving spermatozoa either at 5 degrees Celcius or –196 degrees Celsius involves significant physiological strain on these highly specialized cells (4).

Spermatozoa are particularly vulnerable to cold-induced injury due to their unique structural features including an abundance of polyunsaturated fatty acids in their plasma membranes, little endogenous citosolic antioxidants to protect them from oxidative damage and continual ATP-dependent energy generation necessary to promote motility (5). The primary source of cryoinjury to spermatozoa is the production of reactive oxygen species (ROS), which exceed the limited capability of spermatozoa's antioxidant buffers, resulting in the peroxidation of sperm plasma and mitochondrial membranes, oxidative damage to protein, fragmentation of DNA and the failure of the sperm to maintain normal cellular signaling pathways (6). Similarly, cold shock will induce a phase transition in membrane lipids resulting in membrane architecture damage, loss of selective permeability, early capacitation-like events and mitochondrial dysfunction, all of which reduce post-thawability and fertilizing capabilities of spermatozoa (7).The adverse impact of oxidative stress on the preservation of sperm cells has encouraged a large number of investigations into the protective methods of cryopreservation, especially through the addition of a variety of antioxidant compounds to the media (8). Synthesized antioxidants can protect against oxidative stress, but concerns regarding potential toxicity, residual effects on embryonic development and limitations of regulatory approval have directed attention to antioxidants found naturally in plants (9). The advantages of using plant-based antioxidants include their lower toxicity; the ability to function through a multiple mode of action due to their diverse phytochemical composition; the GRAS classification of many compounds; and consumer acceptance in food-producing animal systems (10). The natural compounds mentioned above utilize various mechanisms in functioning such as directly scavenging reactive oxygen species (ROS), chelating metal ions preventing Fenton reactions, inducing increased expression of endogenous antioxidant enzymes and stabilizing membrane structures (11).

Pomegranates (Punica granatum L.) are a fruit cultivated throughout the Mediterranean, Middle East, and Asia, and have received substantial interest in reproductive biology due to the high concentration of bioactive compounds in the fruit (12). Pomegranates contain a complex mixture of polyphenolic compounds with the majority of the bioactive compounds being ellagitannins (specifically punicalagin and punicalin) along with other polyphenolic compounds, such as ellagic acid, anthocyanidins (delphinidin, cyanidin, pelargonidin), flavonoids (quercetin, kaempferol) and organic acids (13). The bioactive compounds found in pomegranates have potent antioxidant effects through multiple mechanisms: direct scavenging of free radicals as determined by high ORAC values, metal chelation resulting in inhibition of Fenton reactions; increasing expression of endogenous antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase), and reducing inflammation through inhibition of mediators involved in inflammation (14).

This study will address this knowledge gap by investigating the potential of pomegranate seed extract (PSE) as an antioxidant in Tetrahydrofuran Cyclic Ether Yolk (TCFY) extender for maintaining the viability of caprine spermatozoa from epididymal tissue when stored at refrigerated temperature (5°C). A descriptive assessment of multiple quality characteristics (progressive motility, integrity of plasma membrane, viability, morphology) will be conducted at various time intervals (24, 48, 72, 96 and 120 hours) at a variety of PSE concentrations (0%, 5%, 10%, 15% and 20%) will be completed. The information collected will allow for the determination of optimal concentrations of PJ.

Materials and Methods

After obtaining ethical clearance from Institutional Animal Ethics Committee of the College of Applied Medical Sciences, Al-Muthanna University (Iraq), the experiment was carried out in accordance with the animal use ethical guidelines, including minimum suffering to animals and maximum use of biological tissues from animals slaughtered for food. The study was conducted over six months and was completed using three replicate cohorts of different animals to ensure consistent replication of results, and statistically valid results.

Preparation and Collection Sample

Testes were collected from the caudal epididymis of 24 mature male sheep (between 2.5 and 4 years of age, weighing 45 – 55 kg) slaughtered (immediately post slaughter) at a local abattoir in Al-Muthanna Province, Iraq. The selection of animals was based on an inspection for apparent health (no visible lesions), normal scrotal conformation and no abnormalities (as determined after slaughter) of the reproductive tract following inspection after slaughter using an antiseptic solution). The collected testes were excised within 10 minutes after death (after the slaughtering process), and were cleaned of all extraneous connective tissue. The cleaned testes were stored at 37°C in sterile saline (0.9% sodium chloride) (containing 100 IU/mL penicillin G and 100 µg/mL streptomycin sulfate) in order to inhibit any bacterial growth before examination. The storage containers were placed in a cooler insulated box for transport (maintaining a temperature of 35 - 37°C using hot water bottles) to the laboratory within 45 minutes of slaughter. Upon arrival in the laboratory, the testes were rinsed for a short period of time in fresh warm saline (before collection of total epididymal sperm). (15)

The cauda epididymis was dissected from the testis using sterile surgical instruments under aseptic conditions in a laminar flow hood.Isolation was performed by locating and subsequent isolation of the anatomical junction of the corpus and cauda epididymis, and then careful dissection of the cauda region was performed to prevent contamination from blood or any other fluid from the tissue sample. Once isolated, each cauda epididymis was placed in a sterile 100 mm Petri dish, and many small incisions (approximately 15 to 20) were made in the surface of each cauda epididymis with a sterile scalpel blade to permit the passive diffusion of spermatozoa into the TCFY extender medium. To facilitate diffusion, the cauda was incubated in the TCFY extender at 37°C for 10 minutes. After incubation, a sterile pipette was used to collect the sperm suspension from the TCFY extender, and the resulting sperm suspension was filtered through sterile gauze to remove tissue debris. The sperm suspension was then pooled in a sterile centrifuge tube for preliminary evaluation. (16).

Sperm quality was first assessed to ensure that only viable sperm samples were included in the study. A small aliquot (10 µL) of the sperm suspension was placed onto a glass microscope slide and immediately examined using phase-contrast microscopy (400× magnification) to evaluate sperm motility. Sperm with progressive motility were evaluated in a minimum of five microscopic fields. The sperm samples that demonstrated an initial progressive motility of 80% were accepted for subsequent processing. At the same time, the sperm concentration was determined using a Neubauer hemocytometer, appropriately diluted (1:20) in formal-saline solution, and all samples were adjusted to contain 100×10^6 spermatozoa/mL to standardize the treatment groups across the study (17). Local pomegranate fruits (Punica granatum L.) were acquired from a local fruit stand in Al-Muthanna Province during the pomegranate harvest season (September-October). Pomegranates were selected based on uniform size, free of visible defects/decay, and the deepest red colour, which indicates the fruit is at maximum ripeness. The pomegranates were first washed thoroughly with tap water and then again with sterile distilled water, followed by peeling the skin off of each pomegranate. This was done in order to collect the arils from the pericarp of the pomegranate. The seed coats of the pomegranate seeds were removed by manual dissection of the individual seeds from their surrounding pulp and were washed with sterile distilled water to remove excess pulp. The cleaned pomegranate seeds (approximately 200g) were pulped with a small amount of sterile distilled water (1:1 w/v seed to water ratio) by blending them in a high-speed blending machine (2 minutes on the highest speed) in order to extract juice with the minimal production of heat. The blended mixture of pomegranates was filtered through four (4) layers of sterile gauze and further filtered with Whatman No. 1 filter paper to obtain a clear pomegranate extract. This clear pomegranate juice extract was centrifuged for 15000 rcf for 15 minutes at 4°C to separate any remaining particulate matter from the juice extract. The clear supernatant (pomegranate juice extract) was collected in 15ml sterile centrifuge tubes and stored at −20°C until use. Prior to use in each experiment, one sterile centrifuge tube of pomegranate juice extract was thawed by placing it at room temperature for 30 minutes(18) .

Each experiment had a specific design with five different levels of pomegranate juice being added to the TCFY extender: T1, Control (0% PJ); T2, (5% PJ); T3, (10% PJ); T4, (15% PJ); and T5, (20% PJ). The pomegranate extract was added to the base TCFY extender just before the dilution process, thereby reducing the volume of the base extender so that the total final volume would remain constant across treatment groups. For instance, the 10% PJ group was made with 0.9 mL TCFY extender plus 0.1 mL pomegranate extract for a total of 1.0 mL final volume. The pooled sample of sperm was divided into five equal portions, and each portion diluted in its respective treatment extender at a ratio of 1:10 (semen:extender) in order to achieve a standard concentration of 100 million sperm per millilitre.Dilutions of all samples were conducted in a 37°C water bath to prevent thermal shock. After diluting the samples, extended semen was distributed into sterile 2 mL cryovials containing 0.5 mL semen each for all treatment groups so there were multiple vials prepared to retrieve samples from each treatment group at different time points while minimizing repeated warming of the same sample. The vials were placed in a refrigerator and cooled over a 2-hour period from 37 to 5°C by controlling the refrigerator

temperature settings and minimizing thermal shock. The temperature of the samples was maintained at 5°C (±0.5°C) in a temperature-controlled refrigerated area for the storage duration. The temperature of the samples was continuously monitored with a temperature sensor with data logging capability.(19)

Examinations Microscopic

Sperm quality evaluations occurred after the following storage durations: 0 hours (immediately post-dilution), 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. At all storage time points, one vial of semen from each treatment group was removed from the refrigerator and brought to 37°C using a water bath for 5 minutes prior to evaluation. A 10 µL aliquot of the semen sample was placed on a pre-warmed glass slide with a cover slip and evaluated using phase-contrast microscopy at a magnification of 400×. A minimum of 200 sperm were counted across 10 different fields of vision and were categorized as progressively motile (forward moving) or non-progressively motile/immotile **(19)**. The hypo-osmotic swelling test (HOST) was used to assess plasma membrane integrity of the sperm; 0.1 mL semen was mixed with 1 mL of hypo-osmotic solution (100 mOsm/kg, sodium citrate 0.735 g and fructose 1.351 g dissolved in 100 mL of distilled water), incubated at 37°C for 45 min, and then 200 sperm were counted using phase-contrast microscopy (400×) to determine how many sperm had tails coiling or swelling due to the influx of water from the hypo-osmotic solution(20). Live and dead sperm were assessed using eosin-nigrosin staining; equal volumes of semen and eosin-nigrosin stain were mixed and thinly spread out on glass microscope slides, air-dried and evaluated for live and dead sperm using bright-field microscopy at 1000× (oil immersion) magnification. Thinly smeared sperm samples used for eosin-nigrosin staining were also evaluated for morphology; 200 sperm from each of the specimens were evaluated for morphological abnormalities in the head, midpiece, or tail region. (20)

SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) was used to conduct all statistical analyses. Data from 3 replications of this experiment were pooled for analysis. The fixed effect of treatment on each outcome variable was analyzed using one-way ANOVA at each time point. When differences between means were determined using ANOVA to be statistically significant ($P < 0.05$), differences between means were calculated using Duncan's Multiple Range Test. Repeated measures ANOVA was also used to evaluate the overall interaction between treatment, time, and treatment × time on sperm quality characteristics throughout the storage period. Means and standard error of the means (SEM) are reported for all outcomes. Statistical significance was determined at $P < 0.05$. Prior to statistical analysis, all percent outcome data were arcsin transformed to meet the assumption of normality and homogeneity of variances; however, untransformed means are reported in tables and text to enhance clarity.

Results

The effects of different amounts of pomegranate juice on moving sperm from refrigerated male Sheep are summarized in **Table 1**. The statistical analysis showed significant differences ($P<0.05$) between all treatments at all Cold-Preserved Time. At (24) hours after the sperm were cooled, the sperm in the (10%) PJ treatment were the most Progressive motility (87.39) and had much more movement than those in the control (72.31) or the (20,15 and 5%) PJ treatment. After (120) hours, the sperm in the (10%) PJ treatment also had the most movement (68.60) compared to the control (42.88) and the (20%) PJ treatment had the least amount of Progressive motility (13.35).

Table 1. Progressive motility (mean ± SE) of Sheep epididymal spermatozoa supplemented with different concentrations of pomegranate juice at 5°C

	Cold-Preserved Time (hours)				
PJ (%)	**24**	**48**	**72**	**96**	**120**
0	^{C}72.31±1.16	^{C}64.52±1.61	^{C}59.90±1.41	^{C}55.37±1.92	^{C}42.88±1.85
5	^{AB}78.21±1.48	^{A}73.82±1.25	^{B}70.90±1.47	^{B}70.18±1.65	^{B}65.15±0.82
10	^{A}87.39±2.43	^{A}82.64±1.30	^{A}79.97±1.26	^{A}75.65±1.69	^{A}68.60±1.71
15	^{B}81.07±2.66	^{B}76.84±2.34	^{D}46.09±1.00	^{D}43.55±1.05	^{D}34.45±0.66
20	^{D}61.74±1.20	^{C}56.12±0.84	^{E}29.15±1.30	^{E}26.45±1.59	^{E}13.35±0.98

Different superscript letters within the same column indicate significant differences ($P < 0.05$) among treatments.

The plasma membrane of the sperm was evaluated using the hypo-osmotic swelling test, and this test resulted in very different findings on the plasma membrane in all treatments over time **(Table 2)**. The (10%) PJ treatment consistently yielded the highest plasma membrane integrity values throughout the storage time and were 84.90 at (24) hours and 70.27at 120 hours. The (5%) PJ group had significantly better plasma membrane integrity than the control group at almost all evaluation Cold-Preserved Time. The (20%) PJ treatment had the lowest plasma membrane integrity of all treatments by (24,48,72,96 and 120) hours this was less than all other treatments. The

(10%) PJ treatment had comparable plasma membrane integrity to the (5%) PJ treatment at the early evaluation points; however, the (10%) PJ treatment was slightly lower at the later evaluation time points.

Table 2. Plasma membrane integrity (HOST, mean ± SE) of Sheep epididymal spermatozoa supplemented with different concentrations of pomegranate juice at 5°C.

	Cold-Preserved Time (hours)				
PJ (%)	**24**	**48**	**72**	**96**	**120**
0	^{B}72.75±1.21	^{AB}65.55±1.31	^{B}64.35±0.74	^{B}69.35±1.28	^{C}61.05±1.21
5	^{AB}81.58±1.71	^{AB}75.60±1.32	^{AB}71.84±2.15	^{A}70.88±0.82	^{B}68.87±1.74
10	^{A}84.90±1.44	^{A}78.49±1.35	^{A}74.80±1.12	^{A}73.80±1.45	^{A}70.27±1.55
15	^{C}69.80±1.90	^{B}58.15±1.30	^{C}55.44±1.40	^{C}66.45±1.40	^{D}43.62±1.92
20	^{D}57.10±1.78	^{C}52.45±1.22	^{D}42.75±1.02	^{D}28.32±1.75	^{E}23.15±2.43

Different superscript letters within the same column indicate significant differences (P < 0.05) among treatments.

To assess live sperm, the eosin-nigrosin stained cells were counted, and the results from these counts were very similar to those for progressive motility **(Table 3)**. At (24) hours, the (5%) PJ treatment had the highest percentage of live sperm (81.04), and at (120) hours, the (10%) PJ treatment still had a significantly greater percentage of live sperm (73.18) than sperm in the control treatment (54.11) or the (20%) PJ treatment (19.28).

Table 3. Sperm viability (mean ± SE) of Sheep epididymal spermatozoa supplemented with different concentrations of pomegranate juice at 5°C

	Cold-Preserved Time (hours)				
PJ (%)	**24**	**48**	**72**	**96**	**120**
0	^{B}73.20±3.91	^{C}66.50±2.15	^{C}61.95±2.41	^{D}59.68±1.68	^{C}54.11±1.22
5	^{AB}77.16±1.89	^{AB}76.40±2.48	^{A}73.25±2.62	^{B}68.38±1.50	^{B}66.20±2.95
10	^{A}81.04±2.24	^{A}81.92±3.02	^{A}79.52±3.79	^{A}74.09±1.85	^{A}73.18±3.15
15	^{BC}74.32±3.48	^{B}72.64±2.07	^{D}68.85±2.41	^{BC}66.75±2.05	^{D}50.16±1.95
20	^{D}67.81±2.64	^{CD}67.02±2.50	^{E}46.29±1.57	^{E}43.58±2.25	^{E}19.28±1.89

Different superscript letters within the same column indicate significant differences (P < 0.05) among treatments.

The (10%) PJ also afforded a high level of protection for the sperm, with very statistically significant differences from the (0,5,15 and 20%) PJ treatment. Statistical analysis of sperm morphology indicated that moderate amounts of PJ (10%) preserved normal sperm morphology better than the control or (20%) PJ treatment **(Table 4)**. The (10%) PJ treatment had (89.78) normal sperm at (24) hours, and (81.03) normal sperm at (120) hours; however, the (20%) PJ treatment had (17.12) normal sperm at (120) hours.

Table 4. Morphological normality (mean ± SE) of Sheep epididymal spermatozoa supplemented with different concentrations of pomegranate juice at 5°C

	Cold-Preserved Time (hours)				
PJ (%)	**24**	**48**	**72**	**96**	**120**
0	^{CD}75.65±1.06	^{C}72.05±1.52	^{C}68.70±1.80	^{C}65.55±1.84	^{BC}61.03±2.01
5	^{B}78.24±3.61	^{B}74.25±1.28	^{C}69.33±1.91	^{B}69.93±1.36	^{B}63.07±2.49
10	^{A}89.78±1.87	^{A}86.14±1.26	^{A}84.82±1.91	^{A}81.63±1.70	^{A}81.03±3.64
15	^{BC}76.24±2.04	^{C}72.41±1.26	^{BC}70.52±2.64	^{CD}66.35±1.88	^{BC}62.71±1.77
20	^{E}69.14±2.12	^{D}52.97±1.36	^{D}44.63±1.69	^{E}33.87±2.09	^{D}17.12±1.57

Different superscript letters within the same column indicate significant differences (P < 0.05) among treatments.

Discussion

The current study shows moderate doses of pomegranate seed extract (10%) preserve the quality of Sheep epididymal spermatozoa for a longer time period during refrigerated storage at 5°C compared with higher doses (20%) which proved harmful. These findings represent an increased understanding of the complex redox chemistry associated with polyphenolic compounds, and support the need for accurately determining the optimal dosage of polyphenolics when using them in the field of reproductive biotechnology (21). The progressive decline of both motility and viability of spermatozoa in each treatment group during the 120 hour storage period represents the cumulative effect of the many factors associated with the physiological stresses of cooling,

including metabolic suppression, phase transitions in the sperm cell membrane, accumulating oxidative stress, and loss of energy reserves (22).

However, the slower rates of decline, particularly at the 5% and 10% levels of pj, suggest an inhibitory effect on these injurious processes via antioxidant mechanisms. The superior motility of the sperm in the 10% pj group might indicate that this concentration represents an optimal balance between antioxidant protection and maintenance of proper osmolarity, since too high a concentration of an organic solute can cause an osmotic stress on the sperm (23). Maintaining progressive motility is crucial for sperm to have the potential to fertilise an oocyte because it is how sperm are able to move through the female reproductive tract, penetrate the cervical mucus to the oviduct and, ultimately, fertilise an oocyte (24).Protections afforded to motility by pomegranate are based on a complex interplay of different mechanisms that include preserving mitochondrial membrane potential and the ability to produce adenosine triphosphate (ATP), preserving the structure of the axoneme and activity of dynein adenosine triphosphatase (ATPase), preserving the activity of regulatory enzymes that are responsible for controlling motility, and reducing the effects of lipid peroxidation on the membrane rigidity or flexibility that prevents normal flagellar beat patterns (25). Based on the results of a hypo-osmotic swelling test used to evaluate whether the integrity of the plasma membrane was maintained during storage, a 10% concentration of pomegranate juice was the most successful in maintaining sperm plasma membrane function throughout the storage time. The hypo-osmotic stress test (HOST) of spermatozoa evaluated their ability to manage the influx of water into the cell using a hypo-osmotic solution as a reflection of the functional integrity of the membrane transport system as well as the overall health of the membrane (26).

The significant loss of sperm plasma membrane integrity noted for the 20% pomegranate juice group after 72 and 96 h of storage is noteworthy. The results suggest a possible threshold effect whereby excessive concentrations of polyphenolic compounds may switch from being protective to being damaging. The attenuation of the protective effect of high concentrations of polyphenols is speculated to result from several different mechanisms: the pro-oxidant effects of polyphenols in the presence of transition metal ions at high concentrations; osmotic stress related to the concentration of organic solute exceeds the capacity of the membrane transport systems; the possible interaction of high concentrations of polyphenols with membrane lipids, resulting in alterations to the organization of the membrane; or the possibility that high concentrations of polyphenols cause saturation of binding sites on membrane proteins, which results in the alteration of the conformation of the proteins (27). The plasma membrane of motile sperm cells has one of the highest concentrations of polyunsaturated fatty acids, thus oxidative damage occurs primarily at these membranes. Once lipid peroxidation occurs on the plasma membrane, propagation occurs in a chain reaction, disrupting the fluidity, permeability, and proper functioning of membrane proteins (28).Pomegranate polyphenols disrupt the chain reaction of lipid peroxidation through various mechanisms. Punicalagin and ellagic acid directly scavenge lipid peroxyl radicals, chelate pro-oxidant metal ions that catalyze lipid peroxidation, regenerate membrane-associated antioxidants including vitamin E, and interact with the components of cellular membranes to help stabilise the structural integrity of membranes (29).

In this study, the correlation between the maintenance of sperm membrane integrity and preservation of sperm classical motility and viability is consistent with our collective understanding of membrane integrity as a prerequisite for the maintenance of cellular homeostasis, energy metabolism and normal function of the motility apparatus of the sperm (30). Data for sperm viability assessed using eosin-nigrosin staining, which differentiates between live (have intact membranes) and dead (have damaged membranes) sperm cells, corroborated the analysis of sperm membrane integrity and demonstrated a greater protective effect on sperm viability at the 10% pomegranate juice concentration. Close correlations in sperm viability, membrane integrity and motility parameters across treatment groups and time points support the inter-relationship of these three measures, since sperm membrane dysfunction ultimately compromises cellular homeostasis and the energy-dependent processes required for sperm motility (31). The transition from membrane dysfunction to loss of motility and eventual death of the sperm is a pathway common to the deterioration of sperm during storage and emphasises the importance of protecting the sperm membrane as a primary target for intervention (32). The mechanisms by which pomegranate extract protects sperm from loss of viability are likely multiple and may include more than just direct antioxidant activity, such as stabilising membrane structure by means of interactions between pomegranate polyphenols and membrane lipids and proteins, preservation of mitochondrial function and energy metabolism, protecting essential metabolic enzymes from oxidative inactivation and maintaining the ionic gradient across the plasma membrane through salvage of the membrane-associated transport systems (33).
The results of the assessment of morphology indicate that the use of moderate concentrations of pomegranate juice is effective at preventing sperm morphological abnormalities through time of storage while the use of high concentrations leads to an increase in the frequency of sperm morphological abnormalities at long-term times of storage. The occurrence of sperm morphological abnormalities can occur from many mechanisms during cold-

storage of sperm. These mechanisms include membrane blebbing due to cytoskeletal damage; swelling of the acrosome (from osmotic or oxidative damage); coiling or releasing of the tail due to abnormal lipid membrane phase transitions; and decondensation of the nuclear chromatin of sperm nuclei due to oxidative damage to DNA (34). The protective effect on sperm morphology provided by moderate concentrations of pomegranate juice likely involves (at least in part) the preservation of cell cytoskeleton integrity through the maintenance of cellular ATP concentrations as well as the protection of structural proteins from oxidative modification; the inhibition of excessive lipid peroxidation (which is known to create membrane irregularities); and the maintenance of appropriate levels of cellular hydration through osmotic regulation (35). The increased frequency of sperm morphological abnormalities in the 20% pomegranate juice group may be due to osmotic stress through high solute concentration in the solution; pro-oxidant effects leading to sperm structural damage; or an interaction between the polyphenol components of the pomegranate juice and cellular proteins that alter the structural integrity of the sperm (36).

The patterns of all quality parameters (sperm motility, morphology, and viability) showed similar temporal patterns, with all three parameters being relatively stable for approximately 48 - 72 hours post-thaw, followed by more rapid deterioration, and may indicate a critical point or threshold after which cumulative stress to cellular protective mechanisms has been surpassed.This finding supports and reinforces previous studies which have established that as a result of sperm freezing, endogenous antioxidants decrease progressively, metabolic waste products accumulate, and depletes energy stores until oxidative damage reaches a critical level (37) . The functional value of pomegranate supplement usage to delay reaching critical levels is illustrated by 5-10% levels of supplementation. When our data was compared with studies from other researchers, we found some similarities as well as new information. Numerous studies demonstrate the uses of pomegranate extracts in enhancing the preservation of sperm from multiple species; bulls, rams, buffalo, and humans, by supplementing with antioxidants (38). However, the range of optimal concentrations across studies varies widely; from 0.5% to 15% which is attributed to differences in methods of extract preparation, polyphenol composition, and concentration, species sensitivity to the effects of the extract, the base extender, and storage conditions (39). Our results showing a distinct biphasic dose-response relationship, with negative effects noted at 20%, are valuable and demonstrate that optimal concentrations exist that provide benefits that diminish beyond these levels, and provide insight into the development of protocols and the necessity of dose-response studies instead of the assumption that a higher concentration of an antioxidant provides greater protection. The specific superiority of the 10% concentration for maintaining membrane integrity and viability, and motility suggests that the optimal concentration range may vary based on the quality parameter being prioritized and the intended purpose of the preserved spermatozoa (40).

The multiple mechanisms involved with the protective effect of pomegranate during sperm preservation likely reflect the complex chemical nature of the extract. Pomegranate is made up of over 100 unique phytochemicals, and also contains major bioactive compounds, including ellagitannins such as punicalagin and punicalin, ellagic acid and its derivatives, anthocyanins (the pigment providing red color), flavonoids including quercetin and kaempferol, and various organic acids (41). These compounds act synergistically via multiple mechanisms; including directly scavenging reactive oxygen species (ROS) via electron or hydrogen atom donation, chelation of metal ions to prevent Fenton chemistry, stimulation of endogenous antioxidant enzyme expression and activity and anti-inflammatory effects via inhibition of inflammatory mediators, and stabilization of the membrane via interaction with lipids and proteins (38). Much of the relative contribution of the polyphenolic components in contributing to the protection of spermatozoa remains to be determined, although punicalagin has received attention because of its high concentration and strong antioxidant ability. On the whole, the synergistic interactions between multiple constituents likely provide the most protection (4).

Conclusion

Evidence from this research study indicates that the moderate dosages of pomegranate seed extract (10%) improved the preservation quality of elongated Sheep sperm at refrigeration temperatures (5°C), with providing optimal progressive motility preservation and dose providing advantages in terms of viability and membrane integrity preservation. Many detrimental effects occur to membrane integrity with the 20% dose, demonstrating the critical importance of precise dose optimization. The data derived from these research results contribute to the development of evidence-based protocols for improving the preservation of epididymal sperm in small ruminants via the use of natural antioxidants within the framework of assisted reproductive technologies. The data derived from this work will also contribute to improving artificial insemination programs in Sheep, particularly in regions with limited resources where refrigerated storage is more cost-effective than cryopreservation. Future studies should provide additional evidence validating these results through fertility testing, characterization of cellular mechanisms, and optimization for various temperature and duration combinations for sperm storage.

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