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13 **Abstract**

14 The primary cause of sperm quality decline during the freeze-thaw pathway is the peroxidation hazard caused
15 by reactive oxygen species produced by the biological molecules of sperm. Ascorbic acid (Vitamin C) and
16 lycopene are two potent antioxidants that operate to prevent oxidation processes. This study aimed to analyse
17 the effects of ascorbic acid and lycopene on the motility, viability, abnormality and plasma membrane integrity
18 of post-thawed Sapudi rams. Sperm samples were obtained and pooled from six sexually mature Sapudi rams,
19 separated into ten equal proportions and diluted with TEY extender. Semen was supplemented with 0 (C0; L0),
20 1 (C1; L1), 2 (C2; L2), 3 (C3; L3) and 4 (C4; L4) mg/100mL (1–4%) diluent each of ascorbic acid and lycopene,
21 respectively. Total sperm motility, viability, abnormalities and semen membrane plasma (%) were analysed
22 after thawing. C3 and L3 extenders resulted in higher total motility ($p<0.05$) compared to the other extenders,
23 with all treatments higher than that of the control. The extender C3 ($p<0.05$) exhibited the highest semen quality.
24 Finally, the current findings show that C3 and L3 can increase the quality of post-thawed Sapudi ram
25 spermatozoa.

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27 **Keywords** : ascorbic acid; carotenoid; lycopene; TEY; Sapudi ram; vitamin C

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Introduction

A Decree No. Mentan 2839/ KPTS/LB.430/8/2012 designated Sumenep (Madura) as the centre of Sapudi sheep (*Ovis aries*) with a population of more than 16,000 in 2016. Sapudi sheep is the fat-tailed sheep or also known as Javanese fat tailed sheep are native to East Java, Indonesia, which are favourable because they do not involve substantial pens, seem to be low-maintenance, have decent grazing behaviour, eat a variety of grasses, are inexpensive and can be kept supplied with minimal effort. This breed has a natural propensity to have multiple offspring from a single breed. It is possible to increase the current population and genetic performance of a species by embracing the property eligibility (1). Sapudi sheep, along with other native small ruminants, have played an important role in agriculture for centuries, helping alleviate poverty in resource-starved areas of the world. They can adapt to a wide variety of environments, including those that are particularly harsh, cold or arid, and have valuable genetic traits, such as the capacity to survive effectively under minimal input conditions, protection from illnesses and pathogens and more tolerance to heat stress (2, 3). They have the potential to develop as a source of substitute protein for national demand because they require less space and feed than cattle, making them accessible even to the landless (4). In addition, tropical nations like Indonesia are typically separated into multiple small archipelagos spread across the ocean. Effective management of artificial insemination with frozen semen is crucial for the small ruminant sector to become more resilient to increased animal productivity spread evenly throughout the country (5).

Artificial insemination (AI) is the first significant technique used to enhance the genetics and productivity of livestock animals and solve the problem above. As a form of assisted reproductive innovation, the AI method involves the manual insertion of sperm into the uterus of a female to accelerate the fertilisation process and increase efficiency where a small amount of semen can fertilise multiple ewes at once. The use of AI in tandem with other innovations, such as the synchronisation of oestrous and ovulation, can boost the hereditary value of farm animals by increasing the prevalence of high-productivity males (6). It also helps stop the spread of sexually transmitted diseases and allows for the use of males who are deceased, elderly or injured (6). Cryopreserving ram semen and reviewing the state of AI in sheep were both thoroughly discussed (7–10).

Semen cryopreservation is a crucial technique for improving assisted reproductive technologies (ART), especially AI protocols (11). While cryopreservation of ovine spermatozoa can significantly increase the time needed for storage, it also allows and assists their transport over long distances (12), thereby resolving the aforementioned problems. However, on the other hand, in comparison with some species, ram sperms have a higher plasma membrane cholesterol-to-phospholipid proportion. Consequently, ram spermatozoa are more susceptible to cold shock than spermatozoa of other species and have decreased semen quality due to the presence of reactive oxygen species (ROS) (8). Drastic changes in temperature, including cold shock and the formation and solubilization of ice during the freezing-thawing process (13), enhance the production of ROS (14) and are also detrimental to the acrosome, nucleus, mitochondria, axoneme and plasma membrane. To prevent intracellular crystallisation, semen is typically diluted with a preservative extender such as tris egg yolk diluent with a protective agent, including an antioxidant (8). To enhance sperm quality further, a beneficial solution in the form of additional active ingredients is required (15).

Reduced ROS in semen can be eliminated by antioxidants (16) such as vitamin C (ascorbic acid) and lycopene (17, 18). Ascorbic acid prevents intracellular lipid peroxidation by neutralizing the hydroxyl, superoxide and peroxide radicals (19). Lycopene has superior singlet oxygen quenching ability compared to other carotenoids, which accounts for its superior antioxidant activity among carotenoids and its ability to scavenge ROS (20). However, exaggerated usage of antioxidant properties is also recognized to have a negative effect on sperm

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quality, and data on the use of higher antioxidant doses have rarely been reviewed. The purpose of this study was to determine whether the best dosage addition of ascorbic acid or lycopene to the extender improved the freezing resistance of Sapudi ram spermatozoa. This study was the first to focus on the influence of ascorbic acid and lycopene on the motility, viability, abnormality and membrane plasma of frozen-thawed Sapudi ram sperm.

Materials and Methods

Animal experimental design and semen collection

Six normal reproductive Sapudi rams (30-40 kg body weight) were used in this study. Rams were chosen from a flock owned by a traditional farmer in the Sapudi area, Madura East Java, based on their health, and whether they were clinically free of infectious diseases and external or internal parasites. The ejaculates were collected from to 7-8 am twice weekly, and rams were regularly used for semen collection. All rams were individually fed the same concentrate mixture (CP 16%; 2.8%/BW), 10%/BW forage, and kept in individual pens. This research has been registered on ethical clearance with number 082/KE.02/SK/10/2022.

Semen processing and evaluation

The general structure of the sperm was analysed shortly after collection. A Neubauer hemocytometer was used to examine the fresh semen. Ejaculate (100 μL) was transferred to a clean, warm, dry glass slide, observed under a microscope, and scored on a scale from 0 (no motility) to 100 (excellent motility). Spermatozoa viability and abnormalities were defined using a fixed smear stained with eosin, and the percentages of live and dead sperm were estimated (21).

This study analyzed semen with >80% progressive motile spermatozoa and >90% viability for subsequent examination (Table 1). Yolk citrate (2.9% (v/v) sodium citrate dihydrate, 100 ml aquadest mixed with egg yolk 20%, 8% (v/v) glycerol (Merck, Germany), 1.000 IU/ml Penicillin, and 1.000 mg/ml streptomycin) was used as the basic semen diluent (freezing extender). The semen was dissolved to a final concentration of 50 mg/mL. Ten equal aliquots of pooled ejaculate were divided and diluted (37°C) with base extenders containing the antioxidant ascorbic acid (Merck, Germany) (1%, 2%, 3%, 4%, C1, C2, C3, and C4), lycopene (tomato extract lycopene, Merck Germany) (1%, 2%, 3%, 4%, L1, L2, L3, and L4), and two base extenders with no additives as a control for the ten experimental groups (C0/L0). The straws were equilibrated at 5 ℃ for four h. The equilibrated semen was aspirated into 0.25 mL straws and sealed. The straw was frozen in liquid nitrogen vapor (5 cm above liquid nitrogen) for 10 min, and semen was plunged into liquid nitrogen for storage. After storage for 24 h, the straws were thawed individually (at 37°C) for 30 s in a water bath for semen evaluation and all semen samples were immediately examined for sperm quality.

Semen evaluation

This study evaluated sperm performance before and after the freezing procedure to comprehensively evaluate the effects of ascorbic acid and lycopene supplementation and the concentrations, motility, viability abnormalities, and plasma membrane integrity/HOST of spermatozoa before and after freezing. Sperm motility was assessed by homogenization of 10 μL of diluent mixed with NaCl (1:4) and then observed under a microscope (Olympus CH 20). Slide views were taken at ten fields with a magnification of 100 × 400, scores were given in the range of 0-100% on a 5% scale. Eosin staining was used to assess sperm viability. A total of 200 spermatozoa were counted per sample using a light microscope (Olympus CH 20) to differentiate between

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reacted and nonreacted spermatozoa. Dead sperm with damaged acrosomes emitted a robust red colour, whereas non-reacted sperm emitted light pink or no shade. Based on the coiled and swollen tails, a hypoosmotic swelling test was used to determine the functional integrity of the sperm membrane. This was accomplished by incubating 0.1 ml of sperm with 1 ml of a 150 M hypoosmotic solution at 37 °C for 30 min. After incubation, 0.2 ml of the solution was distributed on a warm microscope slide using a coverslip. A magnification of 1000 × was used to examine the 200 spermatozoa under a bright-field microscope. Recorded spermatozoa have an inflated or curled tails (22).

Statistical analysis

Seven replicates were used, and the results were expressed as the mean (SD). All data were analysed using a multifactorial method to determine the effect of ascorbic acid and lycopene supplementation under each condition before and after freezing treatment. Furthermore, the data for each condition were analysed using one-way analysis of variance followed by Tukey's post hoc test to determine significant differences in all parameters between the different groups. In the regression model analysis, a predictive equation was developed, with adjusted supplementation as the dependent variable and sperm quality as the independent variable. SPSS statistical software (version 26.0; IBM Corp., Chicago, IL, USA) (23).

Results

Results evaluation fresh semen

Macroscopic and microscopic analyses are shown in Table 1, and the median sperm concentration in this study was 3.656 × 106 with an average volume of 1.72±0.22 ml. The average mass motility was (+++), with sperms forming massive waves. Moreover, the sperm had an average of 82.14±2.30% progressive motility. The average viability of sperms was 87.02±2.70%. The findings of this study were within the normal range for ram sperm concentration. Sapudi ram sperm abnormalities ranged from 8 to 9%. When the tubes were tilted, the average volume of semen had a creamy color, fresh smell, and moderately thick consistency. The pH of the solution was 6.80. Macroscopic and microscopic quality parameters of fresh sperm (Table 1) were assessed to determine whether the ejaculates were suitable for further processing (24).

The impact of various diluents Ascorbic acid on the cryopreservation of frozen-thaw semen from Sapudi Rams

The addition of different concentrations of ascorbic acid to the diluent significantly increased sperm motility, viability, abnormality, and plasma membrane integrity compared to the control group ($p<0.05$) and made a linear positive graph, with the exception of the addition of 4% ascorbic acid (C4), which started declining sperm quality performance compared to the lower concentration, although still higher than that of the control. Overall, C3 (3% addition of ascorbic acid) significantly showed the most outstanding motility both in freezing treatment or after thawing with the motility (74.14±4.33; 56.00±3.10%), viability (79.29±2.75;63.14±2.47%), abnormality (19.42±1.81;27.14±1.21%), PMI (74.28±1.60;62.85±1.95%) (Table 2, 3) in after dilution and after thawing respectively, except the C4 in PMI after thawing showed the highest results (Table 2).

The impact of various diluents lycopene on the cryopreservation of frozen-thaw semen from Sapudi Rams

The addition of different concentrations of lycopene had the similar pattern with ascorbic acid to the diluent significantly increased sperm motility, viability, abnormality, and plasma membrane integrity compared to the control group ($p<0.05$) and made a linear positively graph, with the exception of the addition of 4% lycopene

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156 (L4), which start declined sperm quality performance compared to the lower concentration even though still
157 higher than the control. Addition of 3% lycopene (L3) also significantly showed the most outstanding motility
158 both in freezing treatment or after thawing with the motility (71.85±4.33; 54.00±3.10%), viability (76.00±2.65;
159 60.14±2.48%), abnormality (18.28±1.79; 25.14±1.21%), PMI (72.29±1.60; 60.85±1.95%) (Table 4-5) in after
160 dilution and after thawing respectively, same with addition ascorbic acid the L4 in PMI after thawing showed
161 the highest results (Table 4).
162
163 *Comparison ascorbic acid and lycopene diluent of semen from Sapudi Rams*
164 Overall, addition of ascorbic acid with same dosage significantly showed have better result than the lycopene
165 addition with C3 significantly showed the most outstanding especially in the result on after thawing data showed
166 in Figure 1.
167
168 *Effect of pre-freezing and after thawing on semen diluted with ascorbic acid and lycopene*
169 The data showed significant differences in pre-freezing and post-thawing in ram semen diluted both with
170 ascorbic acid and lycopene at different concentrations, as shown in Table 6.
171
172 *New approach parameter Δ parameter before freezing and after thawing in Sapudi rams after diluted with*
173 *different antioxidant*
174 This method showed how efficiently the additional additives can maintain sperm quality from damage or
175 general deterioration. The data showed there no significantly different in Δ parameter in percentage (%) before
176 freezing and after thawing in Sapudi rams semen except in ΔPMI, the result showed that addition of lycopene
177 could not preserved the declined the PMI levels before and after thawing mechanism in table 7.
178
179 **Discussion**
180 Sapudi sheep, an indigenous fat-tailed sheep species (*Ovis aries*), is raised as a side business on farms in
181 Indonesia, particularly on East Java Island, because they reproduce easily and can thrive on a restricted diet.
182 Negative effects and inferior reproductive outcomes, such as low semen quality, might result when tethered
183 rams do not receive a nutritionally sufficient diet over a long period (25). The use of frozen semen for artificial
184 insemination (AI) of crossbred sheep has been developed to introduce improved and novel genetics. AI is an
185 essential factor in reproductive control parameters and, in tandem with progeny testing, may improve semen
186 quality by one day through the inclusion of a small amount of additive substrate in the semen diluent (26). The
187 addition of antioxidant substances may serve as an effective strategy to enhance semen cryopreservation
188 procedures in ovine animals (27). Numerous animals and makes have made use of these compounds for this
189 purpose. Instead of blindly extrapolating the results from one animal species to another, it is vital to examine the
190 possibility of an antioxidant benefit. Frozen sperm is unsuitable for routine use because a significant number of
191 spermatozoa undergo changes and become sterile during cryopreservation (28) which was also found in this
192 study (Table 7). Motility and viability decline once sperms are frozen and thawed. The goal of cryopreservation
193 is to maximize the number of post-thawed viable normal spermatozoa that retain their structural integrity,
194 viability, motility, DNA integrity and biological functions associated with fertilization capability. Because of the
195 freezing process, semen loses some of its ability to reproduce after freezing. Though sperm preservation is a
196 cornerstone of ART, its true usefulness has not yet been recognised, as a significant fraction of mammalian
197 livestock sperm loses physiological viability during a freezing and thawing procedure. Cryopreservation of

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198 spermatozoa results in a small percentage of viable cells, and those that survive thawing have a shorter lifespan
199 in the female reproductive system due to damage caused by cold shock. Owing to cold shock, osmotic stress and
200 changes in membrane fluidity and permeability, sperm motility and viability are reduced during
201 cryopreservation (29). Cryopreservation methods have the potential to reduce the antioxidant capabilities of
202 semen (30), ovine semen has natural antioxidants such as GSH, TAC, ALT and AST under normal conditions,
203 but the cryopreservation process depresses these antioxidants by enhancing the production of ROS as the
204 metabolite outcome. This study examined the effect of two antioxidants (ascorbic acid and lycopene) on semen
205 quality. Ascorbic acid and lycopene can produce collagen, proteoglycans and components of the intercellular
206 matrix. The addition of these antioxidants to diluents may improve sperm function by minimizing reactive
207 oxidative damage (31). The addition of 1–4% (1–4 mg/100 mL semen diluent) ascorbic acid and lycopene to
208 cryopreservation settings for Sapudi ram spermatozoa proved neither beneficial nor detrimental to semen
209 performance, particularly at high doses. The results showed that an extra dose of greater than 3% ascorbic acid
210 and lycopene (>3 mg/100 mL semen diluent) resulted in a decrease in sperm quality compared to the lower dose,
211 despite maintaining sperm quality better than the control. However, this suggests that the addition of greater
212 than 3% ascorbic acid or lycopene produces less-effective consequences. Similar mechanisms were observed in
213 the most recent data with the addition of ascorbic acid and lycopene in the same dosage range to bull sperm, but
214 with preservation at 5 ℃ (30, 32). This suggests that further studies using higher doses can be conducted to
215 strengthen the evidence that high doses will lead to a significant decrease in sperm quality.
216 Vitamin C is a potent antioxidant that can be dissolved in water (33). Vitamin C can extinguish hydroxyl,
217 superoxide, and hydrogen peroxide agents while decreasing sperm haemolysis and enhancing tocopherol
218 recycling. The addition of ascorbic acid to the diluent resulted in enhanced spermatozoa and survivability
219 following cryopreservation in some species like bull (34), Awasi ram (35), goat (36) and rooster (37). Vitamin C
220 also lowers the cohesiveness of thawed sperms, thereby facilitating their dissolution (38). These findings also
221 demonstrate that administering up to 3% ascorbic acid to semen might avoid the post-thawing degeneration seen
222 in Tables 2 and 3, which was significantly greater than that of the control. This is because hydroperoxide
223 products, including epoxy fatty acids, alkanes, alkenes, alkanates, hydroxy-alkenals and aldehydes, can be
224 prevented from being formed by cellular oxidative chemicals owing to the ability of ascorbic acid to inhibit their
225 interactions with O_2 and OH (malondialdehyde) (39). Enhanced semen quality in this group was also
226 attributable to the catalysts CAT and GSH, whose levels were increased (40). Furthermore, these results indicate
227 that ascorbic acid preserves cell walls by inhibiting lipid oxidation during both thawing and freezing which is
228 reinforced by the results in C4 which still has the highest PMI, so the application of doses up to 4 mg/100 mL
229 diluent (Table 2 and 3) has a linearly positive effect on PMI. Low ascorbic acid levels (<8 mg/100 mL)
230 encourage the biological synthesis of ROS essential for membrane alteration (17). Antioxidants enhanced the
231 motility of ram sperm the most. Oxidative stress, caused by reactive oxygen species (ROS), is generated during
232 sperm metabolism and reduces sperm viability and fertility. Oxidative stress and lipid peroxidation of sperm
233 membranes may lead to high levels of harmful nitric oxide (NO). Ascorbic acid can directly scavenge,
234 deactivate and repair ROS. Antioxidants decreased lipid peroxidation compared with that in the controls.
235 Incubation enhanced lipid peroxidation because of ROS-induced ATP consumption damage, which hinders
236 sperm motility and membrane integrity (21). Intriguing findings showed that administration of ascorbic acid at
237 dosages >3% caused a reduction in pre-freezing semen quality and worsened post-thawing sperm quality, albeit
238 still providing better data than the control on the entire parameter. This was supported by a similar study in
239 China, which indicated that the addition of >8.5 mg ascorbic acid led to frozen sperm breaking, a result similar

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to that reported in a prior study (31). This may be because ascorbic acid is readily oxidized into inactive dehydroascorbate in strongly oxidative environments or when administered in high amounts (41). Ascorbic acid, a free-radical scavenger, may interact with oxidative stress, and at least eight distinct enzymes have been implicated (e.g., O_2 and OH). Increased doses of ascorbic acid could indeed act as a pro-oxidant in the formation of conversion metal ions (e.g., Fe^{3+}, Cu^{2+}) by providing an electron that reduces such ions to forms that can interact with oxygen substances to form O_2 radicals, increasing the concentration of ROS and a decrease in sperm quality (35) (Figure 1, Tables 2 and 3).

A red pigment, lycopene, is synthesized by vegetation and several microbes (42). Tomatoes contain the highest concentration of this carotenoid, besides watermelon, guava and papaya. Similar to the addition of ascorbic acid, in this study, the addition of lycopene seemed to preserve semen quality before and after thawing better than the control (Tables 4 and 5). This antioxidant has twice the oxygen-scavenging capacity of β-carotene and ten times that of β-tocopherol, making it a potent antioxidant (43). Lycopene neutralises not just hydroxyl radicals but also nitrogen dioxide and hydrogen peroxide. Its lipophilic nature causes it to collect in cell walls and phospholipids, where it exerts a significant effect on the cells themselves. This explained the results of this study which found that the highest addition of lycopene produced the highest PMI data particularly after thawing (Figure 1, Tables 4 and 5). The free radical scavenging properties of lycopene have been previously studied in bull (44) (45), turkey (46) and goat (47). Freezing and thawing protocols in semen can lead to DNA damage, although adding lycopene to the extender can reduce this risk (46). There are potentially three basic mechanisms in which lycopene reacts with free radicals: electron transfer, hydrogen abstraction and radical addition. Another study found that the addition of lycopene to semen diluents is known to improve antioxidant enzymatic activities by reducing ROS generated throughout the semen preservation (43). Lycopene at doses of 1–4 mg/mL considerably ($p<0.05$) improved SOD, CAT and GSH-Px activities and preserved the quality of the sperm. Similar to the results of the present study, experiments with Cashmere goats (47) and bulls (45) stated that the addition of a range of concentrations of 1 and 2 mmol/L lycopene elevated natural antioxidants in semen. Similar to ascorbic acid, a dose >3% culminated in a decrease in sperm quality compared to the lower dosage, despite maintaining sperm quality better than the control. However, this condition suggests that the addition of more than 3% lycopene produces less effective consequences, which is reinforced by a previous study that stated that depending on the concentration, lycopene alters the physical and dynamic characteristics of lipid membranes (44, 45). The stiffness and stability of a lipid membrane can be improved by including polar carotenoids, whereas non-polar carotenoids in high dosages may have a reverse effect (48). The inability of lycopene to maintain spermatozoa stability is attributed to the presence of hydrogen peroxide (H_2O_2), a notable ROS known to quickly escape the ROS-quenching properties of lycopene and to suppress sperm motility through a wide variety of oxidative pathways (45).

Overall, the addition of ascorbic acid at the same dosage showed significantly better results than the addition of lycopene with C3 showing the most outstanding results, especially in the post-thawing data (Figure 1), which may be because, in low amounts, vitamin C is the best antioxidant and more stable than lycopene. Vitamin C supplementation did more than just boost survival rates and safeguarded acrosome and membrane integrity. The addition of vitamin C to diluted semen appeared to protect spermatozoa from DNA damage. When sperm undergo the preservation process, vitamin C can prevent their membranes from rupturing owing to the declining temperature. The addition of vitamin C to ram semen diluent may increase the quality of the sperm because it prevents lipid peroxidation in the plasma membrane. This is reinforced by the results in Table 7 which shows that the administration of vitamin C can significantly maintain PMI compared to that of lycopene, even though

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282 the data on changes (Delta) in other parameters did not show a significant difference in the comparison of these
283 two antioxidants.
284 This new approach adopted in this study could monitor and describe whether the antioxidant in the semen
285 diluent can improve the performance of liquid semen in the final data and determine the effectiveness of the
286 antioxidant on maintaining the quality of liquid semen by screening the decrease occurring in each dose of
287 antioxidant administered. This study also emphasises the fact that the preservation process in frozen semen leads
288 to declined semen quality in Sapudi rams (Tables 6 and 7) due to the damage to sperm that occurs during
289 freezing, which encourages the production of free radicals and decreases sperm quality via redox dysregulation
290 (31). The ROS produced by spermatozoa and leukocytes that infiltrate the semen in the ejaculate are responsible
291 for the dysfunction of mammalian semen. Lipid peroxidation, unsustainable spermatozoa motility and cell
292 nucleus malfunction are the three mechanisms by which free radicals contribute to cell death (49). Oxidative
293 damage is more likely to occur in cryopreserved semen than in fresh ejaculate. Intracellular antioxidant capacity
294 fails to protect against the oxidative stress associated with the harmful effects of ROS upon freezing and
295 thawing (46). The cold shock inflicted on cryopreserved sperm is associated with oxidative damage to critical
296 structural and functional macromolecules, followed by modifications to intracellular signalling pathways and
297 engagement of apoptosis (45). Membranous structures containing large quantities of polyunsaturated fatty acids,
298 sulfhydryl-containing proteins and DNA are extremely susceptible to the cryopreservation process. As a result,
299 improved sperm processing and control methods in sheep breeding may benefit from the addition of ascorbic
300 acid and lycopene to semen diluents. Incorporation of antioxidants at specific doses of ascorbic acid and
301 lycopene can improve the quality of frozen-thawed sperm; the optimal dose of both is 3 mg/100 mL (3%) of
302 diluent. The addition of 3 mg/mL (3%) ascorbic acid resulted in the most significant improvement in post-
303 thawed sperm quality. In addition, supplementation of more than 3 mg/mL ascorbic acid and lycopene appeared
304 to cause a decrease in semen quality although it was still higher than the control.
305

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449 **Tables and Figures**

450 **Tables**

451 **Table 1.** Assesment of Sapudi ram`s fresh semen

Fresh semen assessment	**mean±SEM**	**Normal range for continue semen liquid** (24)
Macroscopic		
Volume (mL)	1.72±0.22	
Colour	Cream	
pH	6.8	
Consistency	Thick	
Microscopic (%)		
Concentration (cell x 10^6)	3,656±9.2	20
Mass motility	+++	++
Motility (%)	82.14±2.30	> 50%
Viabilities (%)	87.02±2.70	80%
Abnormalities (%)	8.9±8.2	<15%
PMI (%)	77.01±2.60	> 60%

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454 **Table 2.** Effect of various ascorbic acid level on sperm quality before freezing

Treatment	Sperm motility	Sperm viability	Sperm abnormality	PMI
C0 (0%)	67.42±2.99[a]	73.86±2.04[ab]	20.57±2.37[b]	66.57±1.39[a]
C1 (1%)	68.29±1.79[ab]	75.71±2.14[bc]	18.57±2.23 [ab]	69.43±1.61[b]
C2 (2%)	70.29±3.15[b]	76.00±2.45[bc]	19.71±1.79[ab]	70.86±1.68[c]
C3 (3%)	74.14±4.34[d]	79.29±2.75[c]	19.43±1.81 [ab]	74.29±1.60[d]
C4 (4%)	72.14±2.27[cd]	77.14±1.77[bc]	20.57±1.61[b]	72.43±1.99[cd]

455 Data show all mean ± standard error of means (n = 7). C0 without addition of ascorbic acids; C1, with addition
456 of 1 mg ascorbic acid into 100 mL extender; C2, with addition of 2 mg ascorbic acid into 100 mL extender;
457 C3, with addition of 3 mg ascorbic acid into 100 mL extender, C4 with addition of 4 mg ascorbic acid into 100
458 mL extender.
459 a-e Means in a column with different superscripts differ significantly at $p < 0.05$.
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Table 3. Effect of various ascorbic acid level on sperm quality after thawing

Treatment	Sperm motility	Sperm viability	Sperm abnormality	PMI
C0 (0%)	50.14±1.57^{ab}	57.43±1.27 ^{ab}	28.57±2.07	58.14±1.77ab
C1 (1%)	52.14±2.27^{bcd}	59.43±2.23 ^{bc}	24.29±2.69	59.86±1.66^{bc}
C2 (2%)	54.43±1.28^{cd}	61.43±2.37 ^{cd}	25.86±2.12	59.00±1.41^{bc}
C3 (3%)	56.00±3.11^{d}	63.14±2.47^{cd}	27.14±1.22	62.86±1.95^{de}
C4 (4%)	53.57±3.21^{bcd}	61.14±1.95^{d}	26.00±1.82	63.57±2.25^{e}

463 Data show all mean ± standard error of means (n = 7). C0 without addition of ascorbic acids; C1, with addition
464 of 1 mg ascorbic acid into 100 mL extender; C2, with addition of 2 mg ascorbic acid into 100 mL extender;
465 C3, with addition of 3 mg ascorbic acid into 100 mL extender, C4 with addition of 4 mg ascorbic acid into 100
466 mL extender.
467 a-e Means in a column with different superscripts differ significantly at $p < 0.05$.
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Table 4. Effect of various lycopene acid level on sperm quality before freezing

Treatment	Sperm motility	Sperm viability	Sperm abnormality	PMI
L0 (0%)	65.14±2.91[a]	71.57±1.99[a]	19.43±2.44[ab]	64.29±1.49[a]
L1 (1%)	66.00±1.63[ab]	73.43±2.07[ab]	17.43±2.23[a]	67.14±1.86[b]
L2 (2%)	68.00±3.06 [bc]	73.71±2.28[ab]	18.57±1.81[ab]	69.71±1.60[c]
L3 (3%)	71.86±4.34[cd]	76.00±2.65[cd]	18.29±1.79[ab]	72.29±1.60[e]
L4 (4%)	69.00±2.23[bc]	76.00±1.73[cd]	19.43±1.62[ab]	70.43±1.98[d]

471 Data show all mean ± standard error of means (n = 7); L0 without addition of lycopene; L1, with addition of 1
472 mg lycopene into 100 mL extender; L2, with addition of 2 mg lycopene into 100 mL extender; L3, with
473 addition of 3 mg lycopene into 100 mL extender, L4 with addition of 4 mg lycopene into 100 mL extender.
474 a-e Means in a column with different superscripts differ significantly at $p < 0.05$.
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478 **Table 5.** Effect of various lycopene acid level on sperm quality after thawing

Treatment	Sperm motility	Sperm viability	Sperm abnormality	PMI
L0 (0%)	48.14±1.57[a]	48.14± 1.57[a]	26.57±2.07[cd]	56.14±1.77[a]
L1 (1%)	50.14±2.27[ab]	50.14±2.27[ab]	22.29±2.69[a]	57.86±1.68[ab]
L2 (2%)	52.43±1.27[bc]	52.43±1.27[ab]	23.86±2.12[bc]	59.00±1.41[b]
L3 (3%)	54.00±3.11[c]	54.00±3.11[bc]	25.14±1.21[bcd]	60.86±1.95[cd]
L4 (4%)	51.57±3.21[bc]	48.14± 1.57[bc]	24.00±1.23[bc]	61.57±2.23[cde]

479 Data show all mean ± standard error of means (n = 7); L0 without addition of lycopene; L1, with addition of 1
480 mg lycopene into 100 mL extender; L2, with addition of 2 mg lycopene into 100 mL extender; L3, with addition
481 of 3 mg lycopene into 100 mL extender, L4 with addition of 4 mg lycopene into 100 mL extender.
482 a-e Means in a column with different superscripts differ significantly at $p < 0.05$.
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485 **Table 6.** Effect freezing and thawing treatment in addition ascorbic acids on sperm quality

Parameter	Motilty	Viability	Abnormality	PIM
Ascorbic acid				
BF (n=35)	70.46±3.79 [a]	76.40±2.79 [a]	19.77±2.06 [b]	70.71±3.09 [a]
AF (n=35)	53.26±3.04 [b]	60.51±2.79 [b]	26.37±2.40 [a]	60.68±2.78 [b]
Δ BF-AF	17.02±0.82	15.89±0.67	-6.60±0.53	10.03±0.70
Lycopene				
BF (n=35)	68.09±3.84 [a]	74.14±2.66 [a]	18.63±2.03 [b]	68.77±3.25 [a]
AF (n=35)	51.85±4.83 [b]	58.31±2.62 [b]	24.37±2.40 [a]	59.09±2.64 [b]
Δ BF-AF	16.23±1.0	15.83±0.63	-5.74±0.53	9.69±0.71

486 Data show all mean ± standard error of means (n = 7); BF: data before freezing treatment, AF: data after
487 thawing treatment.
488 ab Means in a column with different superscripts differ significantly at $p < 0.05$.
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491 **Table 7.** The effect freezing and thawing treatment in addition various ascorbic acids on sperm quality before
492 freezing and after thawing

Parameter	Δ % Motilty	Δ % Viability	Δ % Abnormality	Δ% PIM
C0	25.57±4.31	22.13 ± 2,34	-40.43±19.57	5.71±1.97 [a]
C1	23.43±4.99	21.57 ±2.82	-33.57±28.67	6.43±0.98 [a]
C2	22.43±3.99	19.15 ± 3.67	-32.43±19.58	8.43±2.07[a]
C3	24.14 ±6.62	20.28± 5.09	-40.57±14.91	8.57±1.62[a]
C4	25.86 ± 4.29	20.57 ±3.31	-27.29±15.55	6.59±2.29[a]
L0	25.95 ± 4.46	22.52 ± 2.43	-38.55±21.08	12.63±3.35[b]
L1	23.96 ± 4.51	21.76 ± 3.22	-30.93±29.89	13.83±1.04[bc]
L2	22.77± 3.86	19.34 ±3.50	-29.92±20.51	15.32±3.01[bc]
L3	24.59 ± 6.69	20.75 ± 5.01	-38.59±15.09	15.79±2.85[c]
L4	25.24 ± 4.30	22.14 ±3.41	-24.53±16.09	12.53±3.69[b]

493 Data show all mean ± standard error of means. C0-C4 additional ascorbic acid (1-4%; 1-4 mg/100 ml semen
494 diluent); L0-L4 additional lycopene (1-4%; 1-4 mg/100 ml semen diluent); a-d Means in a column with different
495 superscripts differ significantly at $p < 0.05$.
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Figures

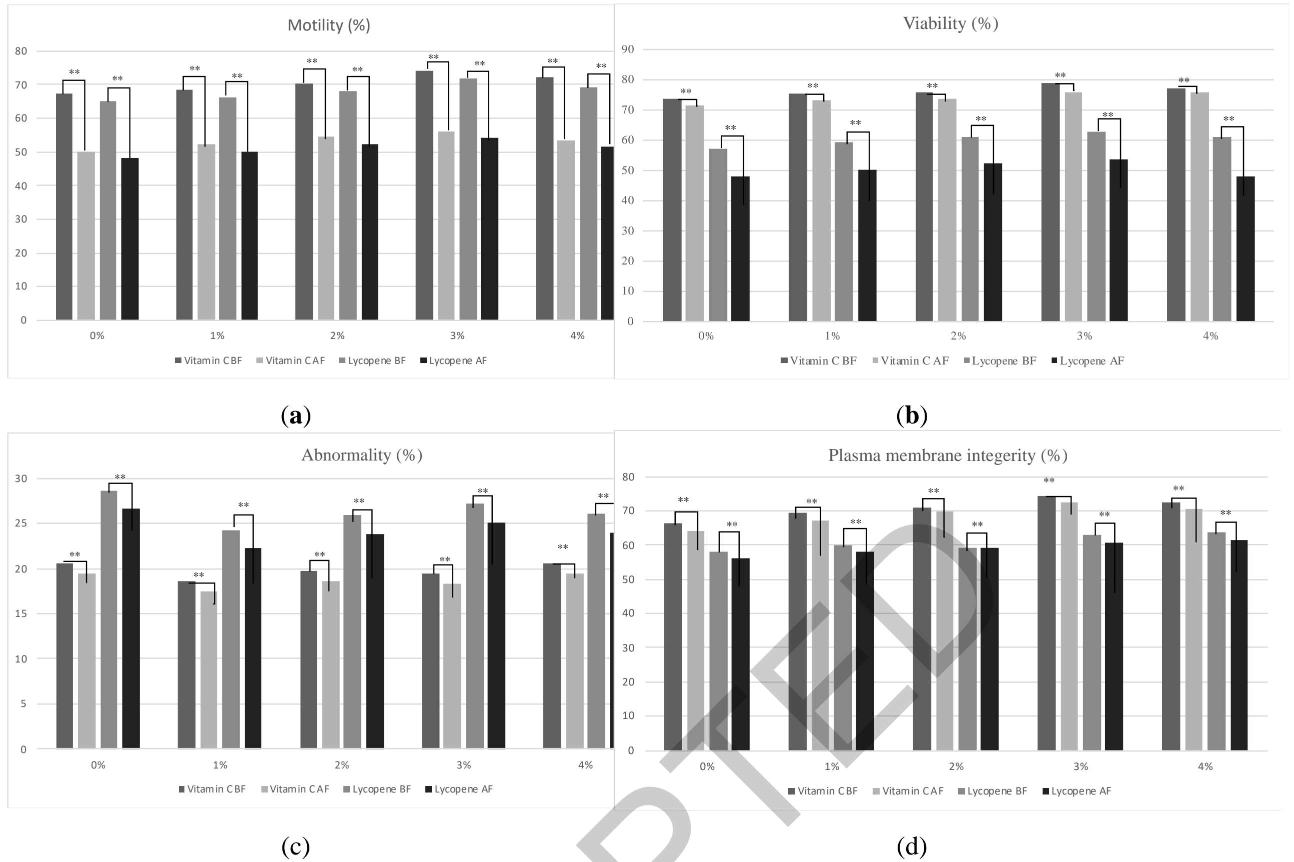

Figure 1. Figure 1. Comparison on semen quality ((a) motility, (b) viability, (c) abnormality and (d) Plasma membrane integrity/PMI) after diluted with ascorbic acid and lycope with the same dosage, the figure described addition of ascorbic acid was better to maintain the semen quality rather than addition of lycopene both in before freezing and in after thawing. The figure also showed declined semen quality ($p<0.05$) in after thawing semen due to the cryopreservation protocols in both group.

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