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Original Article

The Effect of Glutathione (GSH) Supplementation in A Dilution Medium on The Quality of Saanen Goat Semen Stored at 5°C

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Abstract

Objective: This study investigated the effect of Glutathione (GSH) supplementation in a dilution medium on the quality of Saanen goat semen stored at 5°C.
Methods: Semen samples were collected from 1.5-year-old Saanen goats twice weekly using an artificial vagina. Samples with motility >70% were diluted in egg yolk citrate extender and divided into four treatment groups: a control (0 mM GSH), and treatments with 1 mM, 3 mM, and 5 mM GSH in 1 mL egg yolk citrate. Diluted samples were stored at 5°C for 0, 24, 48, and 72 hours. The main variables assessed were sperm motility, viability, and abnormalities. Data were analyzed using a 4x4 factorial ANOVA, with significant differences tested by DMRT at $\alpha = 0.05$.
Results: Results showed that Glutathione supplementation significantly improved sperm motility and sperm viability ($p<0.05$), although no significant effects were observed on sperm abnormalities ($p>0.05$). Mean sperm motility (%) and sperm viability (%) at 0 mM, 1 mM, 3 mM, and 5 mM GSH levels were recorded as 64.61±13.65 and 83.87±8.44; 69.93±13.57 and 88.00±5.40; 72.64±13.26 and 88.57±4.75; and 72.25±13.12 and 86.07±5.69, respectively. The storage time also significantly affected sperm motility and sperm viability ($p<0.05$), with mean for sperm motility (%) and sperm viability (%) at 0, 24, 48, and 72 hours of 82.07±8.78 and 91.27±3.34; 75.25±7.63 and 87.09±5.31; 66.93±8.7 and 84.80±6.37; and 55.17±11.52 and 83.35±9.20, respectively. No significant interaction was observed between Glutathione concentration and storage duration.
Conclusions: Applying 3 mM GSH providing optimal preservation for up to 48 hours

Keywords: Dilution; Glutathione (GSH); Saanen goat; Semen quality.

INTRODUCTION

The Saanen goat, originating from the Saanen Valley in Switzerland, is known for high milk production [1] and its adaptability to tropical environments and feeds [2], making it a promising candidate for artificial insemination (AI) programs in the tropical and subtropical regions, where there is a growing demand for high-yield dairy breeds[3]. However, the major challenges faced during AI, particularly in regions with suboptimal storage infrastructure, is the preservation of semen quality (diluted temperature, storage condition, thawing time, fluctuation, and vibration emission) [4]. Improper thawing

Boer goat semen have been shown to decrease post-thaw motility by up to 35-40% and increase lipid peroxidation as indicated by elevated MDA levels [5]. In other species, such as swine, it has been demonstrated that boar sperm which exposure to vibration stress for 4 hours at 200 rpm reduced sperm viability from approximately 82% to 74% and progressive motility from 62% to 55% [4]. These procedures increase oxidative metabolic activity, making semen susceptible to chemical exposure and triggering free radical formation. It can cause oxidative stress that indicated by an increase reactive oxygen species (ROS) [6].

ROS are free radical compounds derived from oxygen molecules that contain one or more unpaired electrons [7]. ROS originate from membrane systems, including the plasma membrane, mitochondria, and acrosome membrane of spermatozoa [8]. High ROS production in spermatozoa can impair sperm quality. It triggers lipid peroxidation by breaking PUFA bonds in the cell membrane. As a result, mitochondrial function is disrupted, motility is inhibited, and penetration ability is reduced [9] These effects can lead to infertility [10]. Elevated ROS levels also cause harmful increases in cell metabolism and oxygenase activity, which can damage cellular DNA, lipids, and proteins [8].

To counter the detrimental effects of ROS on sperm quality, Glutathione (GSH) -a thiol tripeptide containing a gamma bond with cysteine, carboxyl, and glutamate groups- can be added to the dilution medium [10]. GSH neutralizes free radicals by donating an electron, forming a more stable compound that prevents further oxidation [11]. Studies have shown that GSH enhances antioxidant status and protects ram sperm against oxidative stress during storage [12]. Furthermore, it helps maintain mitochondrial activity [12] and regulate ROS production dynamically [13]. It also improve sperm motility, plasma membrane integrity, and acrosome stability [14]. This study investigates the effect of Glutathione on the quality of Saanen goat sperm stored at 5°C for 72 hours. Understanding its role helps advance reproductive biotechnology and improve breeding efficiency in tropical areas. Practically, this research supports extended semen storage, more flexible AI timing, and better reproductive outcomes in Saanen goat systems.

MATERIALS AND METHODS

Semen collection

This study was conducted from January to March 2023 at the Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta. Semen was collected using an artificial vagina twice weekly in the morning, for a total of seven collections. Semen quality was evaluated for suitability for processing, with fresh semen characteristics shown in Table 1.

Table 1. Characteristics of Fresh Saanen Goat Semen

Parameter	Mean ± SD
Volume (mL)	1.25 ± 0.35
Colour	Cream
pH	6.8
Smell	Distinctive
Consistency	Thick
Concentration (x 10^7/mL)	521.57 ± 262.44
Motility (%)	88 ± 4.04
Viability (%)	82.42 ± 3.76
Abnormalities (%)	6.28 ± 1.11

Preparation of dilution medium

This study used an egg yolk citrate extender, composed of buffer and egg yolk at a 1:4 ratio[11]. The buffer solution contained 2.9 grams of sodium citrate dihydrate dissolved in 100 mL of distilled water. The buffer and egg yolk mixture was homogenized with 20 mL egg yolk, 8 mL glycerol (Merck, Germany), 1,000 IU/mL penicillin, and 1,000 mg/mL streptomycin [15]. Glutathione was added at concentrations of 0 mM (0 mg/mL, control), 1 mM (0.0001 g/mL), 2 mM (0.002 g/mL), 3 mM (0.0003 g/mL), and 5 mM (0.0015 g/mL) to the extender. The semen-to-extender ratio was set at 1:10 [16] using a proportion of 0.01 μL semen to 1 mL egg yolk citrate extender.

Semen evaluation

The equilibration process was conducted at a temperature of 5°C, with evaluations carried out at storage intervals of 0, 24, 48, and 72 hours. At each interval, microscopic

assessments were performed on the diluted semen to evaluate sperm motility, viability, and abnormalities, using a microscope and advance observer (Optilab, India) with magnification 10 or 40 times [17]. Slide views were captured at ten different fields using a 100 x 400 magnification, and ranging from 0% to 100%. Sperm viability was evaluated using eosin staining. To distinguish between reacted and nonreacted spermatozoa, a light microscope (Olympus CH 20) was used to count 200 spermatozoa per sample. While non-reacted sperm emitted pale pink or no shade, dead sperm with damaged acrosomes released a powerful red color.

Data analysis

Data were analyzed using a 4x4 factorial analysis of variance (ANOVA) with 7 repetitions. Significant results were further tested with Duncan's Multiple Range Test (DMRT) at a 5% significance level (α = 0.05) and presented in Mean±SD.

RESULTS

Motility

The additions of Glutathione and storage duration are affected on the sperm motility. Analysis results showed that different concentrations of Glutathione and storage duration had a significant effect on sperm motility ($p<0.05$) In general, the averages of sperm motility toward to Glutathione concentration and the day storage were 64.61±13.65 and 82.07±8.78; 69.93±13.57 and 75.25±7.63; 72.64±13.26 and 66.93± 8.70; and 72.25±13.12 and 55.17±11.52 respectively. These results can be seen in Table 2.

Table 2. Sperm Motility with Glutathione Addition at Different Storage Times

Day storages	Glutathione Concentrations				Mean
	T0	T1	T2	T3	
0 hour	75.29±12.42	81.86 ± 7.84	86.14±5.34	85.00±4.43	82.07±8.78^{a}
24 hours	71.29± 8.36	76.29 ± 6.55	76.29±6.55	77.14±9.03	75.25±7.63^{ab}
48 hours	61.86± 8.47	66.29 ± 8.54	70.43±8.50	69.14±8.57	66.93± 8.70^{b}
72 hours	50.00±10.00	55.29±13.12	57.71±12.39	55.17±11.18	55.17±11.52^{c}
Mean	64.61±13.65^{x}	69.93±13.57^{y}	72.64±13.26^{y}	72.25±13.12^{y}	

^{abc} Different superscript in the same column indicated significant differences ($p<0.05$) on long day storages; ^{xy} Different superscript in the same row indicated significant differences ($p<0.05$) on Glutathione.

T0 = added of 0 mM Glutathione (control); T1 = added of 1 mM Glutathione; T2 = added of 3 mM Glutathione; T3 = added of 5 mM Glutathione.

Viability

The addition of Glutathione and storage duration are affected on the sperm viability. Analysis results showed that different concentrations of Glutathione and storage duration had a significant effect on sperm viability ($p<0.05$). In general, the averages of sperm viability toward to Glutathione concentration and the day storage were 83.87±8.44 and 91.27±3.34; 88.00±5.40 and 87.09±5.31; 88.57±4.75 and 84.80±6.37; 86.07±5.69 and 83.35 ±9.20 respectively. These results can be seen in Table 3.

Table 3. Sperm Viability with Glutathione Addition at Different Storage Times

Day storage	Glutathione Concentrations				Mean
	T0	T1	T2	T3	
0 hour	88.43 ± 3.98	92.50±4.21	93.57±2.07	90.57 ± 3.12	91.27±3.34^{a}
24 hours	85.00 ± 6.11	88.64±6.52	88.14±3.29	86.29 ± 5.35	87.09±5.31^{b}
48 hours	81.36 ± 8.18	86.00±5.17	86.79±5.60	85.07 ± 6.56	84.80±6.37^{bc}
72 hours	80.43±15.51	84.86±5.69	85.78±8.08	82.35 ± 7.53	83.35 ±9.20^{c}

Mean	83.87±8.44^{x}	88.00±5.40^{y}	88.57±4.75^{y}	86.07±5.69^{xy}

abc Different superscript in the same column indicated significant differences ($p<0.05$) on long day storages; xy Different superscript in the same row indicated significant differences ($p<0.05$) on Glutathione.

T0 = added of 0 mM Glutathione (control); T1 = added of 1 mM Glutathione; T2 = added of 3 mM Glutathione; T3 = added of 5 mM Glutathione.

Abnormalities

The addition of Glutathione and storage duration are not affected on the sperm abnormalities. Analysis results showed that different concentrations of Glutathione and storage duration had not a significant effect on sperm abnormalities ($p>0.05$).

In general, the averages of sperm abnormalities toward to Glutathione concentration and the day storage were 3.59±2.27 and 3.73±1.71; 4.09±2.08 and 3.35±2.35; 3.64±1.71 and 3.68±2.01; 3.32±2.05 and 3.88±2.07 respectively. These results can be seen in Table 4.

Table 4. Sperm Abnormalities with Glutathione Addition at Different Storage Times

Day storages	Glutathione Concentrations				Mean
	T0	T1	T2	T3	
0 hour	3.64±1.46	3.14±0.90	3.64±1.82	4.50±2.42	3.73±1.71
24 hours	2.91±3.19	3.64±2.70	3.64±2.12	3.21±1.55	3.35±2.35
48 hours	3.43±1.51	5.21±2.64	3.79±1.22	2.29±1.55	3.68±2.01
72 hours	4.36±2.72	4.36±1.21	3.50±1.96	3.29±2.32	3.88±2.07
Mean	3.59±2.27	4.09±2.08	3.64±1.71	3.32±2.05	

The addition of glutathione at the different storage times are not significant differences ($p>0.05$) on sperm abnormalities.

T0 = added of 0 mM Glutathione (control); T1 = added of 1 mM Glutathione; T2 = added of 3 mM Glutathione; T3 = added of 5 mM Glutathione.

DISCUSSION

The quality of diluted semen declines rapidly during processing, causing cell destabilization and osmotic pressure changes that result in decreased sperm motility. According to Amtiran et al. temperature changes during processing reduce the permeability of the sperm cell membrane to calcium ions. Osmotic pressure changes can also initiate plasma membrane rupture in spermatozoa. Herrera et al. found that osmotic pressure causes ion loss, leading to dehydration, cell shrinkage, and membrane damage, thereby decreasing motility [18]. Additionally, semen processing triggers oxidative stress, indicated by increased ROS, which Andersen et al. reported as accelerating apoptosis in cells. The adverse effects of ROS can be mitigated by adding antioxidants, such as Glutathione, to the extender[19].

The addition of Glutathione as an external antioxidant source can prevent oxidative damage and maintain sperm motility. It also stated by Ribeiro noted that Glutathione protects disulfide bonds, helping to preserve sperm motility and reduce ROS, which in turn limits lipid peroxidation in the plasmalemma, intracellular enzyme loss, and chromatin damage [20]. In this study, a 3 mM Glutathione concentration yielded optimal sperm motility results. This is consistent with Ogata et al. who found that Glutathione at concentrations of 2 – 5 mM did not damage frozen-thawed Canine sperm integrity, while 6 – 7 mM increased osmotic pressure, potentially causing sperm tail breakage and reducing motility, acrosome integrity, and mitochondrial function [21]. Similarly, Solihati et al. reported that 6 mM and 8 mM Glutathione only maintained Local Ram motility at 43.82±4.07% and 46.83±3.58%, respectively [22].

Sperm motility declines as storage time increases, likely due to mechanical damage to macromolecules essential for metabolism and organelle protection. Pasyah et al. indicated that decreased sperm motility during storage

results from membrane structure deterioration, disrupting sperm metabolism [23]. Meliyana et al. also attributed sperm motility decline to ROS formation during storage[24]. Consistent with Solihati et al. sperm metabolism generates hydrogen peroxide (H_2O_2), which degrades into hydroxyl radicals. Tethool et al. stated that sperm motility decreases during storage due to lactic acid accumulation from sperm activity, which becomes toxic in excess and further reduces sperm motility [25].

No significant sperm motility decline was observed after 24 hours of storage, likely due to nutrient supply from the extender, which gradually depletes over time. This finding aligns with Zuhdi and Ducha who observed that DEG sperm motility declines correlate with limited energy availability required for metabolism during cold storage; as energy supply decreases, sperm fibril contraction stops, reducing motility [26]. Sperm motility continued to decrease after 48 hours of storage, though it was still adequate for AI use. Shi et al. reported a significant decrease in progressive Ram sperm motility (54.2±2.00%) for Ram sperm supplemented with 50 mM Glutathione after 72 hours of storage [12]. Manehat et al. found that Bali cattle motility significantly decreased at 96 hours (45 ± 5.00%) attributed to the loss of lipoproteins and lecithin necessary to maintain plasma membrane integrity [27]. According to Bergstein-Galan et al. Ovine sperm stored at 5°C showed decreased sperm motility but maintained Ovine sperm viability, making it suitable for insemination up to three days [28].

Sperm viability in the control group significantly decreased over the storage period. During storage, sperm metabolic activity remains relatively high, making the cells vulnerable to membrane damage and lactic acid accumulation. According to He et al. sperm viability declines due to limited energy reserves, damage to the acrosome and plasma membranes, and pH reduction that promotes lactic acid formation [29]. Herrera et al. noted that membrane damage impacts the semipermeable membrane's ability to regulate the entry and exit of substances in the plasma[18]. Rachmawati et al. stated that membrane deterioration reduces macromolecular function, affecting the control and movement of Ca^{2+} ions into the cytosol, which in turn decreases viability [30].

The addition of Glutathione has proven effective in protecting the acrosome from free radical damage and maintaining acrosome membrane stability. Gangwar et al. found that supplementing the extender with 2 mM Glutathione shields the acrosome from oxidative damage and helps maintain membrane stability [31]. Moreover, Glutathione supplementation at the right concentration preserves mitochondrial integrity, thus reducing ATP synthesis decline and sustaining sperm viability. Gangwar et al. reported that adding 0.5 mM Glutathione to the extender medium increased cell survival by 13% and maintained Murrah bull sperm mitochondrial integrity [31]. However, higher Glutathione concentrations resulted in lower Saanen sperm viability percentages, possibly due to toxicity. According to Ogata et al. observed that excessively high Glutathione concentrations, such as 7 mM, caused membrane swelling and reduced mitochondrial integrity, thereby decreasing Canine sperm viability[21]. Muthmainnah et al. suggested that high Glutathione concentrations could be toxic, as the high sulfur content in Glutathione lowers the solution's pH in seurukan fish Osteochilus vittatus sperm [32]. He et al. reported that an excessively low pH can lead to sperm death due to excessive lactic acid build up [29].

The analysis indicated that storage duration significantly affects sperm viability ($p<0.05$), with longer storage times leading to greater viability reduction. According to Pasyah et al. extended storage results in excessive electrolyte concentrations that dissolve the lipoprotein coating on sperm cell walls, changing membrane permeability and causing cell death [23]. Glutathione addition effectively slowed the sperm viability decline, as noted by Zuhdi and Ducha who reported that Glutathione supplementation in the extender maintained DEG sperm viability at 50% up to the fourth day [26]. Shi et al. found that adding antioxidants to the extender reduced the negative impact of cooling on

total Ram sperm viability in sheep, preserving membrane functionality when stored at 5°C for 24 hours [12].

The addition of Glutathione and varying storage durations of Saanen goat semen at 5°C did not have a significant effect on sperm abnormalities ($p>0.05$). Observations of the effect of Glutathione addition and different storage durations on sperm abnormalities (Table 4). Sperm abnormalities are influenced by genetic factors and temperature changes. Lei et al. noted that Glutathione supplementation does not reduce abnormalities, as the cooling process can cause irreversible changes to the Ram sperm plasma membrane, including glycoprotein loss and abnormal energy metabolism [12]. Solihati et al. found while Glutathione affects sperm metabolism, it does not alter Local Ram sperm morphology [22]. According to Manehat et al. sperm abnormalities are due to cold shock, which disrupts osmotic pressure balance as a result of ongoing metabolic processes [27]. This finding aligns with Solihati et al. who reported that adding 0 mM, 4 mM, 6 mM, 8 mM, and 10 mM Glutathione yielded no significant differences in Local Ram abnormalities, with values of 3.19±0.46, 2.75±0.31, 2.60±0.45, 2.45±0.51, and 2.50±0.53, respectively [22].

Pasyah et al. found that fresh semen stored for 0, 24, 48, and 72 hours also showed no significant differences in abnormalities, with values of 8.00±1.00, 7.67±0.81, 7.50±1.04, and 25.83±6.34, respectively [23]. The average of abnormality rate for Saanen goat spermatozoa ranges from 3-4%, which is considered normal (below 10%). According to Solihati et al. high-quality sheep and goat semen typically has an abnormality rate of less than 15%. In AI, the total number of motile spermatozoa per insemination is more critical than the percentage of abnormalities [22]. The number of sperm abnormalities reaching under 20% will not affect fertility [33].

Direct artificial insemination (AI) trials using Glutathione (GSH)-supplemented semen straws in goats remain limited. This aligns with Dhara et al., who noted that the application of AI in small ruminants is still not widely adopted, partly due to its origin as a modified protocol from bovine practices [34]. However, Kim et al. demonstrated the successful implementation of AI in Korean black goats using frozen–thawed semen straws, achieving a pregnancy rate of 42.9% when AI was conducted 48 hours after estrus synchronization. This finding supports the practical feasibility of AI programs in goats using preserved semen. [35]. Therefore, improving semen quality through antioxidant supplementation—such as Glutathione—could be a valuable strategy to enhance fertility outcomes when combined with optimized estrus synchronization and precise insemination timing.

CONCLUSION

The addition of Glutathione concentration and storage duration had a significant effect on sperm motility and sperm viability but did not significantly affect sperm abnormalities. A Glutathione concentration of 3 mM resulted in the highest sperm motility and viability. Sperm quality was maintained up to 48 hours of storage. No interaction was observed between Glutathione concentration and storage duration.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest relevant to this article.

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