Research Article

Frozen Semen Quality of Kalang Buffalo Using Lycopene

Rizkie Elvania[1], Tintin Rostini[1], Sakiman[2], Ani Susilawati[2], Emilda Adriani[2], Abd Malik[1]*

[1]Department of Animal Science, Faculty of Agriculture, Islamic University of Kalimantan Banjarmasin, South Kalimantan, Indonesia; [2]Center of Artificial Insemination Office, Province, South Kalimantan-Banjarbaru, Indonesia.

Abstract | An important factor to successful of artificial insemination (AI) in buffalo was the quality of spermatozoa post-thawed. Therefore, research was conducted on the use of lycopene as an effort to improve the quality of spermatozoa post-thawing. This study aimed to assess the impact of incorporating lycopene into both the skim milk and egg yolk extender at varying doses on pre-freeze and post-thawed Kalang buffalo semen. Four Kalang buffalo, aged between 6 to 7 years and weighing 500-550 kg, were involved in this investigation. Semen collection was conducted weekly a 12- week period using an artificial vagina. Lycopene was added to the skim milk-egg yolk diluent at concentrations of 1%, 2%, 3%, and 4%, while the control group received no lycopene substitution. The freshly collected semen underwent macroscopic and microscopic evaluations. Subsequently, the semen was assessed pre-freezing and post-thaw with parameters such as viability, motility, abnormality, and plasma membrane integrity. The findings revealed that the inclusion of 1% and 2% lycopene in the diluent before freezing exhibited significantly higher ($P < 0.05$) spermatozoa motility. Moreover, the viability and motility of post-thawed semen significantly improved ($P < 0.05$) at lycopene doses of 1% and 2%. The addition of 1% to 2% lycopene in the extender demonstrated a positive effect on the sperm qualities, particularly enhancing the viability and motility of post-thaw semen from Kalang buffalo.

Keywords | Kalang buffalo, Lycopene, Motility, Post-thawed, Spermatozoa, Viability

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***Correspondence** | Abd Malik, Department of Animal Science, Faculty of Agriculture, Islamic University of Kalimantan Banjarmasin, South Kalimantan, Indonesia; **Email:** sidol_99@yahoo.com
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INTRODUCTION

The swamp buffalo, known as the Kalang Buffalo (*Bubalus bubalis carabauesis*) in the Hulu Sungai Selatan district, is among the original germplasms of South Kalimantan Indonesia. It's crucial to continuously develop and preserve the Kalang buffalo due to their significant role in the socio-economic life of breeders. According to data from the Statistical Center of South Kalimantan Province, the population of buffalo livestock is currently on a decline (Malik *et al*., 2018). Artificial insemination is one method that could be utilized to increase the population of cattle and buffalo. Artificial insemination (AI) serves as an essential and effective tool for genetic enhancement in various livestock species, including buffalo, horses, cattle, goats, sheep, and poultry (Malik *et al*., 2017). One crucial factor influencing the success of artificial insemination (AI) is the quality of post-thawed spermatozoa, which is controlled by the composition of the cryopreservation medium. According to Sheikholeslami *et al*. (2020), the freezing process poses a risk to spermatozoa, causing damage to the sperm cell membrane due to cold shock. Additionally, the thawing process in frozen semen may elevate the production of reactive oxygen species (ROS), subsequently impacting the activity of antioxidant enzymes in semen plasma (Ferramosca and Zara, 2022; He *et al*., 2016).

The use of antioxidants in cryopreservation is serves as a strategy to counteract oxidative stress, thereby mitigating damage to plasma and acrosome membranes and reducing levels of reactive oxygen species (Sangeeta *et al*., 2015; Amidi *et al*., 2016). Among the elements used to enhance the quality of post-thawed sperm is lycopene. Lycopene, a polyene hydrocarbon with an open unsaturated acyclic chain containing 13 double bonds-11 of which are conjugated and arranged linearly-serves as an effective antioxidant (Saputra, 2019). The addition of lycopene to the semen diluent of cattle, sheep, and turkeys showed significant results on DNA integrity, acrosome integrity, and motility, and could reduce sperm lipid peroxidation (Sheikholeslami *et al*., 2020; Rosato *et al*., 2012; Tuncer *et al*., 2014; Bucak *et al*., 2015). As well as enhance the qualities of spermatozoa in the cryopreservation of ram semen (Zou at al., 2021).

Data on the potential utilization of lycopene in buffalo semen is limited. Therefore, we aimed to assess the effect of lycopene added to the skim milk-egg yolk extender at various doses before freezing and post-thaw semen of Kalang buffalo.

MATERIALS AND METHODS

Collection of semen

Semen samples were collected from four fertile buffalo bulls using an artificial vagina. These buffalo bulls, aged 6 to 7 years and weighing 500-550 kg, were housed at the Artificial Insemination Center in Banjarbaru, South Kalimantan with temperate 75-90°F, and humidity 80% (3.44°S 114.84°E, 28m asl. 91°F. 13:000). Each buffalo was kept in individual cages and provided with uniform feed content. Pasturage accounted for around 10% of their body weight, while each bull received 2-3 kg/head/d of concentrate, contained 16% crude protein and 2.6% crude fat. Water was available *ad libitum*. Semen was in the mornings at approximately 8:00 am once weekly for 12 weeks. Immediately after collection, each ejaculate was transported to the laboratory for quality assessment. Only samples exhibiting motility above 70%, with abnormality and viability levels surpassing 80%, were utilized for the study. This research adhered to ethical standards and was approved by the Institutional Animal Care and Use Committee at the Faculty of Agriculture, Islamic University of Kalimantan, with the protocol number 005/UC/2023.

Design of research

The primary diluent in this study were comprised 10% skim milk, 5% egg yolk, 8% glycerol (Merck, Darmstadt, Germany), 1% fructose (Scharlau, Barcelona, Spain), along with 1000 IU/mL penicillin and 1000 mg/mL streptomycin. The research aimed to evaluate the effects of various concentrations of lycopene (Puritans Prite INC. Holbrook, NY, USA) with the following treatments; doses of 0 (control), 1, 2, 3, and 4% of lycopene were added to 100 mL of the skim milk and egg yolk extender. After macroscopic and microscopic evaluation, the buffalo semen evaluated for quality, then semen was adjusted to achieve a final concentration of 25 x 10^6 sperm/mL. Subsequently, the aqueous semen was loaded into 0.25 mL straws (Biovet, France). These straws were positioned horizontally on a cold rack (4-5°C) and then suspended approximately 4 cm over the nitrogen surface to expose them to liquid nitrogen vapor (-5°C) for 10 minutes. Following this, the straws were gradually immersed and stored in liquid nitrogen. Each treatment group's straws were preserved for a week, with five straws selected from each treatment in every replication, thawed at 37°C for 30 seconds, and analyzed.

Evaluation of spermatozoa

Viability, motility, and abnormality assessments were conducted on fresh, pre-freezing, and post-thaw semen samples. Spermatozoa motility was examined using a tiny droplet (10 µL) from new, pre-freezing, and post-thaw semen. A small drop was placed at the center of a slide, covered with a cover slip, and observed under a phase contrast microscope at 400x magnification. Viability and abnormality were assessed according to the methods outlined by Sheikholeslami *et al.* (2020), wherein sperm in four distinct microscopic fields, comprising at least 200 sperm, were evaluated following the protocol established by Memon *et al.* (2012). The membrane integrity of sperm was evaluated by using a hypo-osmotic swelling test (HOST) to assess curled and swollen tails, as described by Kaka *et al.* (2015). Assessment of sperm membrane integrity performed both pre-freezing and post-thawing.

Statistics analysis

The research data concerning the addition of varying levels of lycopene to the extender was assessed using analysis of variance (ANOVA) via the linear model implemented in SAS (SAS 9.1, 2001). Data averages were evaluated as mean ± SD, with significance set at $P < 0.0$

RESULTS AND DISCUSSION

The macroscopic and microscopic findings for fresh semen are detailed in Table 1.

The motility of spermatozoa from Kalang buffalo, stored at 2°C for 4 hours before freezing add of 1% and 2% lycopene, exhibited significantly higher ($P < 0.05$) rates compared to the control (P0). While, the use of 3% and 4% lycopene resulted in considerably lower motility. In contrast, assessing sperm membrane integrity after storage at 2°C for 4 hours before freezing showed no significant differences across all

lycopene doses compared to the control. However, there was a declining trend in membrane integrity observed clearly at 1% and 2% lycopene concentrations (refer to Table 2).

Table 1: The qualities of fresh semen Kalang Buffalo.

Parameters	Mean ± SD
Volume of semen/ejaculate(mL)	3.82±1.80
Color	Cream
pH	7.00±0.21
Motility (%)	76.21±2.20
Viability (%)	86.09±0.91
Abnormality (%)	9.81±3.54
Sperm concentration (ml)	1.370 x 10^6±1.34

Table 2: Effect of lycopene on motility, viability, abnormality, and membrane integrity of before freezing Kalang buffalo spermatozoa.

Lycopene in 100 mL extender(%)	Viability (%)	Motility (%)	Abnormality (%)	Membrane integrity (%)
P0 (control)	59.23±1.32	51.04±0.15[a]	17.42±2.40	50.06±2.02
P1(1)	61.07±2.10	58.16±2.11[b]	15.09±1.81	51.16±1.90
P2(2)	60.16±0.76	56.27±1.80[b]	14.61±0.92	52.25±2.71
P3(3)	57.31±1.83	39.21±0.91[c]	15.32±1.39	49.19±1.53
P4(4)	55.27±1.36	37.53±3.17[c]	13.21±2.70	47.28±0.61

[a,b,c] Values in the same column with superscript differences shown significant differences at P<0.05 (n=12).

The process of freezing sperm can be successful if there is good recovery of sperm post-thawed. The results indicated that post-thawed viability and motility add of 1 and 2% lycopene were significant (P<0.05) compared to control and others. Meanwhile, viability and motility post-thawed at 3 and 4% of lycopene were clear (P<0.05, Table 3). Furthermore, sperm membrane integrity post-thaw showed no significance between the control and all treatments. The sperm abnormality pre-freezing and post-thawed was insignificant between the control and all treatments.

Table 3: Effect of lycopene on motility, viability, abnormality, and membrane integrity of post-thawed Kalang buffalo spermatozoa.

Lycopene in 100 mL extender (%)	Viability (%)	Abnormality (%)	Membrane integrity (%)
P0 (control)	51.16±0.58[a]	17.31±1.90	41.25±2.11
P1(1)	56.15±1.93[b]	16.13±2.14	42.31±2.90
P2(2)	57.25±2.10[b]	15.91±0.93	43.19±0.91
P3(3)	41.14±2.53[c]	12.50±0.72	39.21±1.27
P4(4)	36.31±1.48[c]	14.13±1.58	38.14±0.54

[a,b,c] Values in the identical column with superscript differences shown significant differences at P<0.05 (n=12).

Evaluated fresh semen showed good results and was feasible to be processed into the freezing process (Table 1). Overall, the results were strengthened by Yendraliza *et al.* (2022) and Malik *et al.* (2018). Furthermore, Bauer and Nixon (2020) highlighted the potential physical damage and dysfunction caused sperm by freezing due to the excessive generation of reactive oxygen species (ROS). In this study, the addition of 1% and 2% lycopene to the extender before freezing showcased a notably enhanced protective effect on sperm motility. This finding is consistent with in-vitro observations on poultry and turkey semen. Previous research has also indicated the positive impact of lycopene on sperm motility, attributing its ability to reduce ROS and safeguard sperm from oxidative damage (Babaei *et al.*, 2021; Rosato *et al.*, 2012).

Post-thawed sperm viability and motility were essential factors because spermatozoa must swim from the vagina to the uterus until the site of fertilization. Therefore, efforts were needed to increase good viability and motility in post-thaw sperm. The addition of 1 and 2% lycopene at the extender increased the viability and motility of buffalo semen post-thawed. The increased sperm motility and viability post-thawed at this dose might be attributed to the action of lycopene as an antioxidant to trap loose radicals and stop the chain reactions (Bohm *et al.*, 2003), reducing the ROS and easing oxidative stress, thus averting oxidative harm to proteins, lipids, and DNA (Al-Mutary, 2021; Warraich *et al.*, 2020; Palozza *et al.*, 2012). The results were in line with those stated that the addition of lycopene could increase the post-dilution viability of sperm of sheep or goats (Bucak *et al.*, 2009), banteng (Sarıözkan *et al.*, 2015), dogs (Sheikholeslami *et al.*, 2020), and rabbits (Babaei *et al.*, 2021).

On the other hand, doses of 3 and 4% lycopene showed a tendency to decrease the viability and motility of sperm compared to controls. It was suspected that at 3-4% lycopene in 100 ml of the extender, it has reached a saturation point, which has an impact on decreasing the viability and motility of post-thawing buffalo sperm. These results were similar to Kandelousi *et al.* (2013) who reported that if the antioxidant level was high, it would cause a lessen in motility, sperm concentration, and morphological changes.

The membrane integrity of sperm was of key essential during the freezing until thawed process, and the extender has to protect it against cryopreserved damages. In this study, no significant difference was observed in the membrane integrity of sperm before freezing and after thawing between the treatments and the control group. However, a noticeable trend towards increased membrane integrity was noted especially at 1-2% lycopene concentrations. This observation aligns with the findings of Akhtar *et al.*

(2010, 2022), who reported that the membrane integrity of stallion and buffalo sperm showed no significant difference following cryopreservation

CONCLUSIONS AND RECOMMENDATIONS

The addition of 1 to 2% lycopene in 100 mL of skim egg yolk milk can increase the viability and motility of Kalang buffalo spermatozoa post-thawed. Therefore, the addition of lycopene up to 2% per 100 mL of skim milk-egg yolk is recommended.

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NOVELTY STATEMENT

The innovation in this research was to focus on the potential of lycopene as an antioxidant in a diluent to improve the quality of swamp buffalo sperm post- thawed

AUTHOR'S CONTRIBUTION

Rizkie was head of the Project, A Malik design and write a draft of the article, Tintin R and Ani S collect and analysis data, and Emilda and Sakiman were rearing of experimental livestock.

Conflict of interest

The authors have declared no conflict of interest.

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