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Influence of Melatonin as an Antioxidant on the Preservation of Liquid Ram Semen in Tris-Fructose Egg Yolk Diluent

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ABSTRACT

Excessive free radicals can induce oxidative stress, leading to the inhibition of phosphorylation processes that supply energy for sperm capacitation and the acrosome reaction in spermatozoa during fertilization. This study investigated the impact of incorporating the antioxidant melatonin into the tris-fructose egg yolk diluent on the quality of ram liquid semen preserved at 4℃. Fresh semen with ≥70% motility was diluted in the tris-fructose egg yolk group (control), tris-fructose egg yolk with 0.5 mM melatonin (TFEY + M0.5), tris-fructose egg yolk with 1 mM melatonin (TFEY + M1), tris-fructose egg yolk with 2 mM melatonin (TFEY + M2). Semen was stored in the refrigerator at 4℃ for 5 days (D0–D5). The motility, viability, abnormality, and plasma membrane integrity of the semen were evaluated every 24 hours. The results indicated no significant difference (P>0.05) between the control group and the liquid semen treatment at D0. Liquid semen quality was significantly improved with melatonin treatment compared to the control group. The results of this study indicate that TKTF + M0.5 had the highest motility and viability values and can maintain abnormality values. The TKTF + M2 group had a higher plasma membrane integrity value than the other treatment groups.

Keywords*:* free radicals, melatonin, ram, tris-fructose, egg yolk

ABSTRAK

Stres oksidatif akibat radikal bebas yang berlebihan dapat memicu terhambatnya proses fosforilasi. Fosforilasi berperan menyediakan energi bagi spermatozoa untuk kapasitasi dan reaksi akrosom pada saat membuahi sel telur. Tujuan penelitian ini dilakukan untuk mengevaluasi pengaruh penambahan antioksidan melatonin pada pengencer tris-fruktosa kuning telurterhadap kualitas semen cair domba yang disimpan pada suhu 4 ℃. Semen segar dengan motilitas ≥70% kemudian diencerkan pada kelompok tris kuning telur fruktosa (kontrol), tris kuning telur fruktosa dengan melatonin 0,5 mM (TKTF + M0,5), Tris kuning telur fruktosa dengan melatonin 1 mM (TKTF + M1), tris kuning telur fruktosa dengan melatonin 2 mM (TKTF + M2). Semen disimpan di dalam *refrigerator* bersuhu 4 ℃ selama 5 hari (H0–H5). Semen dievaluasi motilitas, viabilitas, abnormalitas dan keutuhan membran plasma setiap 24 jam. Hasil penelitian menunjukkan tidak terdapat perbedaan nyata (P>0,05) antara semen cair kontrol dan perlakuan pada H0. Kualitas semen cair dengan perlakuan melatonin memiliki nilai lebih tinggi dibandingkan kontrol. Hasil penelitian ini menunjukkan TKTF + M0,5 memiliki nilai motilitas dan viabilitas tertinggi serta dapat mempertahankan nilai abnormalitas. Kelompok TKTF + M2 memiliki nilai keutuhan membran plasma lebih tinggi dibandingkan kelompok perlakuan lainnya.

Kata kunci: domba, melatonin, radikal bebas, tris-fruktosa kuning telur

INTRODUCTION

The development of livestock in Indonesia is facilitated by a tropical climate that promotes the growth of forage plants necessary for feed requirements. Forage is a crucial feed source for farm animals, particularly ruminants, as it constitutes 70% of their total feed intake, with the remaining 30% being concentrate (Saputro et al., 2018). Sheep are small ruminants that can be farmed due to their dual role as a source of animal protein and an economic resource (Pewitasari & Bastoni, 2019). Consequently, it is essential to increase the sheep population.

Reproductive technology of Artificial Insemination (AI) is an effort to increase the sheep population. Artificial insemination involves the introduction of sperm cells into the reproductive tract of healthy females utilizing an insemination device (Mahalubi et al., 2019). Kusumawati and Leondro (2014) assert that AI can lower costs by eliminating male involvement, preventing infectious diseases, and enhancing genetic quality. AI in sheep presents challenges due to the anatomical structure of the female reproductive system, which is small and convoluted, complicating the placement of spermatozoa (Inounu, 2014). The semen, whether frozen or liquid, must be of high quality to enhance the potential for successful artificial insemination.

According to Susilawati et al. (2016), utilization of frozen semen is associated with low sperm fertility and a 30% mortality rate of sperm due to the extreme freezing process. AI with liquid semen is easier and cheaper because it only needs to be stored in a 4–5 °C refrigerator (Zuhdi and Ducha, 2022). Low temperatures can reduce spermatozoa quality due to cold shock; therefore, a diluent is needed to protect spermatozoa during low-temperature storage (Wiratri et al., 2014).

The tris-fructose Egg yolk diluent comprises a tris buffer solution, egg yolk, and fructose. Egg yolk contains lecithin and lipoprotein, an anti-cold shock agent. Fructose serves as an energy source. The decline in spermatozoa quality during storage can also be caused by the presence of free radicals in the form of Reactive Oxygen Species (ROS), which can cause damage to the spermatozoa membrane structure and trigger inhibition of the phosphorylation process (Blegur et al., 2020). Therefore, the application of antioxidants as inhibitors of free radicals is essential. One of the antioxidants that can be used is melatonin. Melatonin functions as an antioxidant by capturing singlet oxygen and enhancing the activity of endogenous antioxidant enzymes. These enzymes act as free radical scavengers, decreasing the levels of electrons that leak during ATP formation, thereby minimizing the production of ROS.

MATERIALS AND METHOD

The ingredients used were fresh sheep's semen, tris (hydroxymethyl)-aminomethane (MERCK, Merck kGaA, Germany), citric acid monohydrate (MERCK, Merck kGaA), D(+)-Fructose anhydrous (MERCK, E. Merck, Germany), chicken egg yolk, melatonin, penicillin-G (Meiji, PT Meiji Indonesian Pharmaceutical Industries, Indonesia), streptomycin sulfate (Meiji, PT Meiji Indonesian Pharmaceutical Industries, Indonesia), lubricant (KY-Jelly), warm water (50–55 °C), 0.9% physiological NaCl, 70% alcohol, eosin nigrosin dye, 3% NaCl (MERCK, Merck kGaA, Germany), trisodium citrate and D(-) fructose (MERCK, E. Merck, Germany) as hypo-osmotic swelling (HOS) media, distilled water, and mili-Q water.

Preparation of Diluent Ingredients

A total of 0.271 g/mL tris (hydroxymethyl) aminomethane, 0.14 g/mL citric acid, and 0.1 g/mL fructose were dissolved in 10 mL of Milli-Q water and combined with 20% egg yolk. All ingredients were homogenized and subsequently supplemented with penicillin at a concentration of 1000 IU/mL and streptomycin at 1 mg/mL for a volume of 0.05 mL. The resulting diluent was categorized into four groups: tris-fructose egg yolk (control), tris-fructose egg yolk with 0.5 mM melatonin (TKTF + M0.5), tris-fructose egg yolk with 1 mM melatonin (TKTF + M1), and tris-fructose egg yolk with 2 mM melatonin (TKTF + M2).

Semen Collection

Semen was collected utilizing an artificial vagina. Semen was collected from sexually mature ram exhibiting libido and in optimal physical condition. The collection utilized an ewe as bait. The collector positioned himself to the right and parallel to the rear of the bait ewe, adjusting his stance according to the dimensions of the ewe's body. Following the male's mounting of the female, the erect penis was positioned into the artificial vagina by adjusting the preputium to facilitate ejaculation. The penis remained in the artificial vagina until the male dismounted. The artificial vagina was positioned downward and rotated in a figure-eight motion. The collected semen was promptly transported to the laboratory for analysis.

Fresh Semen Evaluation

The microscopic examination included assessments of motility, viability, concentration, abnormalities, and the integrity of the plasma membrane (IPM). The motility examination was conducted by placing a drop

of semen on a glass slide and covering it with a glass cover slip. The examination was conducted using a light microscope at a magnification of 40×10. Motility assessment involved comparing progressively motile spermatozoa with those exhibiting non-progressive movements, such as backward motion or spinning. The assessment was presented as a percentage ranging from 0% to 100%.

Viability and abnormality checks were carried out with eosin nigrosin staining. Two microliters of semen and eosin nigrosin solution were homogenized in a 1:1 ratio, subsequently examined on a glass slide, and dried using a heating plate. Observations were conducted from eight fields of view using a light microscope at a magnification 40×10. Live spermatozoa are characterized by a colorless head, whereas dead spermatozoa exhibit a colored head. The live and dead spermatozoa were subsequently counted manually with a counting device. Viability was calculated by dividing the number of live spermatozoa by the total number of spermatozoa multiplied by 100%.

The abnormality examination was carried out using the same method as for viability. Observations were made from eight fields of view and under a light microscope at a magnification of 40×10 times. The percentage of abnormality was calculated by dividing the number of live spermatozoa by the total number of spermatozoa multiplied by 100%. In the concentration test, the semen was diluted 500 times with 3% NaCl, then homogenized and dripped into a Neubauer chamber using a micropipette. Observations were made by counting the number of spermatozoa that entered the five boxes of the Neubauer chamber using a 40×10 magnification light microscope.

The Hypo-Osmotic Swelling Test (HOST) was employed to evaluate the integrity of the sperm plasma membrane. The test involved incubating a mixture of semen and HOST solution (comprising 0.9 g of fructose and 0.49 g of sodium citrate dissolved in 100 ml of distilled water) at 37°C for 5 to 30 minutes. The sample was subsequently placed on a glass slide, covered with a glass cover, and examined using a microscope with 40×10 magnification. A sperm exhibiting a circular tail demonstrates an intact plasma membrane, whereas a sperm with a straight tail displays an incomplete plasma membrane.

Semen Preservation

The semen utilized had a motility of 70% or greater. The semen was diluted using an tris-fructose egg yolk diluent, to which melatonin was incorporated at 0.5 mM, 1 mM, and 2 mM, resulting in a final spermatozoa concentration of 100 million cells/ml. The diluted semen was subsequently stored in a refrigerator at 4°C for 5 days.

Evaluation of Liquid Semen

Following dilution, the liquid semen was promptly assessed as H0. The evaluation was conducted daily for 24 hours, labeled as H1-H5. The evaluation parameters included motility, viability, abnormality, and the integrity of the plasma membrane of spermatozoa.

RESULTS AND DISCUSSION

The evaluated fresh semen had a motility value of 80.71 ± 3.45, viability of 83.28 ± 2.28, abnormality of 2.57 ± 0.53, intact plasma membrane of 81.00 ± 3.31 and spermatozoa concentration of 3510.7 ± 180.19. The motility, viability, abnormality, and plasma membrane values of fresh semen in this study were considered normal (Husnurrizal *et al*., 2021).

The examination results at H0 indicated no differences in motility, viability, abnormality, and intact plasma membrane values among the control group and the groups TKTF + M0.5, TKTF + M1, and TKTF + M2 (Table 1). Following several days of storage, a decline was observed in the percentage of all parameters, except abnormality, which exhibited an increase. The decrease resulted from the diminishing availability of energy substances and the declining stability of the buffer solution. Sperm motility declined daily (Table 2). The values for TKTF + M0.5, TKTF + M1, and TKTF + M2 showed no significant differences during storage ($P>0.05$). The control values, TKTF + M2, TKTF + M0.5, and TKTF + M1, commenced a decline at H1. TKTF + M0.5 had the highest motility value compared to the other treatments. The study by Septiani et al. (2017) reported a daily decrease in the motility of preserved sheep semen. A cold shock from low-temperature storage can lead to membrane damage by altering the typical configuration of the sperm plasma membrane to a hexagonal form (Ariantie et al., 2014).

Moreover, membrane damage may lead to the

Table 1 Evaluation Results of Fresh Semen

Parameters	Results
Motility (%)	80.71 ± 3.45
Viability (%)	83.28 ± 2.28
Abnormalities (%)	2.57 ± 0.53
Intact Plasma Membrane (%)	81.00 ± 3.31
Spermatozoa concentration (million cells/ml)	3510.7 ± 180.19

Table 2. The motility of spermatozoa following a 5-day storage period in a tris-fructose egg yolk diluent with varying concentrations of melatonin

Parameters	Motility of spermatozoa storage on day-					
	H0	H1	H2	H3	H4	H5
Control	80.7±1.8^{aA}	70.0±5.7^{aB}	58.5±3.7^{aC}	51.4±6.9^{aD}	40.0±7.0^{aE}	17.8±8.5^{aF}
TKTF + M0.5	82.8±2.6^{aA}	77.8±2.6^{bA}	71.4±3.7^{bB}	64.2±5.3^{bC}	55.7±5.3^{bD}	37.8±11.1^{bE}
TKTF + M1	82.8±2.6^{aA}	77.8±4.8^{bA}	68.5±8.9^{bB}	62.5±9.1^{bB}	52.8±11.1^{bC}	37.1±9.5^{bD}
TKTF + M2	83.5±2.4^{aA}	77.1±3.9^{bAB}	71.4±3.7^{bB}	64.2±6.0^{bC}	55.7±7.8^{bD}	32.8±9.9^{bE}

Note : Small letters superscripts (a, b) differ in the same column significantly (P <0.05). Capital letters superscripts (A, B, C, D, E, F) differ in the same row significantly (P <0.05). Control: fructose egg yolk tris; TKTF + M0.5: fructose egg yolk tris with 0.5 mM melatonin; TKTF + M1: fructose egg yolk tris with 1 mM melatonin; TKTF + M2: fructose egg yolk tris with 2 mM melatonin.

release of the aspartate aminotransferase (AST) enzyme, which is involved in ATP production within cell mitochondria and subsequently enters seminal plasma. This causes disruption of ATP production and decreases motility value. Table 2 indicates that the motility percentages for TKTF + M0.5, TKTF + M1, and TKTF + M2 exceed that of the control group. The TKTF + M0.5 treatment exhibits the highest motility value at H5 compared to the other treatment groups.

Viability refers to the proportion of live to dead spermatozoa. Live spermatozoa exhibit a colorless head, whereas dead spermatozoa are identified by a colored head. Rahman et al. (2018) found that the viability of sheep spermatozoa decreases as storage time increases. Literature indicates that the decline in sperm viability is attributed to several factors: a reduction in nutrient availability for metabolic energy due to prolonged storage time, the decrease in the ability of diluent materials to protect sperm from cold shock, and a decrease in pH resulting from lactic acid accumulation during metabolism (Butta et al., 2021).

Table 3 indicates no significant difference (P>0.05) between the control and TKTF + M0.5 and TKTF + M2 at H2. Additionally, the comparison between the control and TKTF + M1 and TKTF + M2 at H5 also showed no significant difference (P>0.05). The viability values of the control, TKTF + M0.5, TKTF + M1, and TKTF + M2 decreased at H1. The values for TKTF + M0.5, TKTF + M1, and TKTF + M2 showed no significant differences (P>0.05) throughout the storage period. The TKTF + M0.5 treatment exhibited the highest viability percentage among the treatments analyzed (Table 3).

Abnormality serves as a parameter for evaluating sperm quality, as irregular cell structures can lead to complications during fertilization. Abnormalities are typically categorized into primary and secondary types. This study identified several abnormalities, including broken tails, missing heads, coiled tails, small heads, and double heads. Ismaya (2014) identifies primary abnormalities as small heads and double heads, whereas secondary abnormalities encompass broken tails, circular tails, and missing heads. The proportion of abnormalities rose over the course of five days of storage (Table 4). The rise in spermatozoa abnormalities is attributed to cold shock and ongoing metabolic processes during storage at 3–5 °C, leading to an osmotic pressure imbalance.

Significant differences (P <0.05) in abnormality values were observed between the control and the groups TKTF + M0.5, TKTF + M1, and TKTF + M2 over the 5-day storage period. The differences in values among TKTF + M0.5, TKTF + M1, and TKTF + M2 were not statistically significant (P > 0.05) throughout the storage period. Control abnormality, TKTF + M0.5, TKTF + M1, and TKTF + M2 increased at H1. TKTF + M0.5 had the lowest percentage of abnormality compared to the other treatments. The percentage of sperm abnormalities was reduced with the addition of melatonin compared to its absence.

The HOST method was employed to evaluate the sperm membrane's integrity. This method is used to determine whether the sperm membrane is intact. An intact membrane is characterized by a circular sperm tail, whereas an incomplete membrane is denoted by a non-circular or linear sperm tail. The sperm plasma membrane's integrity diminishes daily during storage (see Table 5). Prolonged lipid peroxidation results from excessive oxygen consumption, which decreases the plasma membrane's integrity (Hariono et al., 2022). In addition, excessive oxygen consumption also causes damage to the plasma membrane, loss of enzyme function and membrane transport.

There was a significant difference (P<0.05) between the control and TKTF + M2 values for plasma membrane integrity during storage. The values for the control group and the TKTF + M0.5 and TKTF + M1 groups did not show significant differences (P > 0.05) at H3 and H4. The differences in values among TKTF + M0.5, TKTF + M1, and TKTF + M2 during storage were not statistically significant (P>0.05). The TKTF + M2 value exhibited the highest percentage of plasma membrane integrity. The integrity of the sperm plasma

Table 3. The viability of spermatozoa following a 5-day storage period in a tris-fructose egg yolk diluent with varying concentrations of melatonin

Parameters	Motility of spermatozoa storage on day-					
	H0	H1	H2	H3	H4	H5
Control	84.0±2.8^{aA}	71.7±2.8^{aB}	67.0±2.7^{aC}	58.8±5.6^{aD}	50.7±4.0^{aE}	40.4±5.7^{aF}
TKTF + M0,5	85.5±1.3^{aA}	78.1±3.0^{bAB}	71.2±5.2^{abBC}	66.0±8.6^{bCD}	59.7±9.4^{bDE}	53.7±9.7^{bE}
TKTF + M1	85.7±2.8^{aA}	78.8±3.5^{bB}	74.0±3.6^{bB}	67.7±4.3^{bC}	60.1±7.4^{bD}	49.7±9.5^{abE}
TKTF + M2	83.5±2.4^{aA}	77.1±3.9^{bB}	71.4±3.7^{abB}	64.2±6.0^{bC}	55.7±7.8^{bD}	49.1±8.2^{abD}

Note : Small letters superscripts (a, b) differ in the same column significantly (P <0.05). Capital letters superscripts (A, B, C, D, E, F) differ in the same row significantly (P <0.05). Control: fructose egg yolk tris; TKTF + M0.5: fructose egg yolk tris with 0.5 mM melatonin; TKTF + M1: fructose egg yolk tris with 1 mM melatonin; TKTF + M2: fructose egg yolk tris with 2 mM melatonin.

Table 4. The abnormality of spermatozoa following a 5-day storage period in a tris-fructose egg yolk diluent with varying concentrations of melatonin

Treatment	Spermatozoa abnormality on day-					
	H0	H1	H2	H3	H4	H5
Control	2.5±0.7^{aA}	5.0±1.5^{bB}	6.5±1.3^{bC}	8.0±1.5^{bD}	9.8±1.3^{bE}	11.0±1.1^{bE}
TKTF + M0.5	2.1±0.3^{aA}	3.7±0.7^{aB}	5.0±1.1^{aC}	6.1±1.0^{aD}	7.4±0.9^{aE}	8.4±0.9^{aF}
TKTF + M1	2.1±0.6^{aA}	3.4±0.9^{aAB}	4.7±1.1^{aB}	6.2±1.3^{aC}	7.2±1.3^{aCD}	8.5±1.5^{aD}
TKTF + M2	2.1±0.6^{aA}	3.5±0.9^{aB}	5.1±1.3^{aC}	6.7±1.3^{aD}	7.8±1.3^{aDE}	9.1±1.6^{aE}

Note : In the same column, the same small letters in superscript (a, b) differ in a statistically significant manner (P<0.05). Capital letters in superscript (A, B, C, D, E, F) exhibit a statistically significant difference in the same row (P<0.05). Control: fructose tris-yolk; TKTF + M0.5: fructose tris-yolk with 0.5 mM melatonin; TKTF + M1: fructose tris-yolk with 1 mM melatonin; TKTF + M2: fructose tris-yolk with 2 mM melatonin.

Table 5. The integrity of the sheep sperm plasma membrane following a 5-day storage period in a tris-fructose egg yolk diluent with varying concentrations of melatonin

Treatment	MPU of spermatozoa on day-					
	H0	H1	H2	H3	H4	H5
Control	82.2±2.3^{aA}	65.2±7.6^{aB}	58.5±4.7^{aC}	49.7±2.7^{aD}	45.8±4.6^{aD}	33.5±3.3^{aE}
TKTF + M0.5	83.1±2.2^{aA}	76.7±1.7^{bB}	67.2±7.4^{bC}	57.2±6.0^{abD}	50.8±5.3^{abE}	43.5±6.9^{bF}
TKTF + M1	81.7±2.2^{aA}	77.0±3.1^{bA}	66.8±8.1^{bB}	57.4±8.0^{abC}	51.1±5.3^{abC}	43.8±9.0^{bD}
TKTF + M2	83.2±1.7^{aA}	77.7±3.0^{bA}	69.7±8.9^{bB}	61.5±8.5^{bC}	55.1±7.3^{bC}	45.7±7.0^{bD}

Note : In the same column, the same small letters in superscript (a, b) differ in a statistically significant manner (P<0.05). Capital letters in superscript (A, B, C, D, E, F) exhibit a statistically significant difference in the same row (P<0.05). Control: fructose tris-yolk; TKTF + M0.5: fructose tris-yolk with 0.5 mM melatonin; TKTF + M1: fructose tris-yolk with 1 mM melatonin; TKTF + M2: fructose tris-yolk with 2 mM melatonin.

membrane, with the addition of melatonin, remained above 40%. The values for H5 are as follows: TKTF + M0.5 was 43.57 ± 6.92, TKTF + M1 was 43.85 ± 9.04, and TKTF + M2 was 45.71 ± 7.01 (Table 5). The integrity of the plasma membrane influences spermatozoa viability, characterized by the ratio of plasma membrane integrity to viability, which shows no significant difference (Ardhani et al., 2020). The findings of this study indicate that there is no significant difference between plasma membrane integrity and viability (see Table 3 and Table 5). Damage to the membrane in the head of the spermatozoa results in staining, which enhances its viability. Conversely, damage to the midpiece, particularly the mitochondrial membrane, leads to reduced motility, as the mitochondria serve as the primary site for ATP production necessary for sperm movement (Hastuti et al., 2020).

Spermatozoa will continue to metabolize to produce energy during the storage process. Sperm metabolism may generate lactic acid, which can be detrimental to membrane integrity if produced excessively. Furthermore, sperm metabolism generates free radicals. Reactive oxygen species, or free radicals, are reactive oxygen molecules that can induce cell injury via lipid peroxidation. Lipid peroxidation has the potential to damage

sperm plasma membranes and mitochondrial DNA. Lipid peroxidation leads to oxidative damage of unsaturated fatty acids, destabilizing the membrane as the fatty acid chain cleaves, subsequently forming a toxic compound that can harm the cell membrane. Reactive oxygen species can easily damage the sperm plasma membrane because unsaturated fatty acids are susceptible to oxidation. A diluent was added to protect and maintain the quality of the sperm during the storage process. The diluent used in this study was tris egg yolk supplemented with fructose. Tris functions as a buffer solution and maintains osmotic pressure and electrolyte balance. As one of the ingredients in the tris yolk diluent, citric acid serves to disperse the fat granules in the egg yolk to facilitate the observation of spermatozoa movement under a microscope. Widjaya (2011) asserts that egg yolks provide amino acids, carbohydrates, vitamins, and minerals essential for the survival of spermatozoa. The yolk is an extracellular cryoprotectant containing lipoproteins and lecithin that protect the integrity of sperm cells during storage at 5 °C by maintaining the integrity of the lipoprotein envelope (Swari et al., 2019).

The incorporation of fructose into the diluent is intended to facilitate ATP energy production in spermatozoa. Fructose is a monosaccharide carbohydrate that is quickly metabolized by spermatozoa (Purbiandara et al., 2016). Muhammad et al. (2018) indicate that the simplicity of this metabolic process is attributed to the shorter metabolic pathway of fructose, specifically through fructolysis. Fructose is efficiently metabolized by ketohexokinase (KHK) into fructose-1-phosphate (F1P). Fructose-1-phosphate is subsequently cleaved by aldolase B (AldoB) into dihydroxyacetone phosphate (DHAP) and glyceraldehyde. The latter is then phosphorylated by triokinase (TrioK) to yield glyceraldehyde 3-phosphate (GAP), which ultimately generates ATP (Lee & Cha, 2018). In addition, fructose can protect spermatozoa from *cold shock* during storage at 4°C. The protective effect arises from the hydrogen bond established between the hydroxyl group of the sugar and the polar phospholipid head group of the cell plasma membrane. Subsequently, the sugar will occupy the position of the water molecule during the dehydration process and storage (Mukminat et al., 2014).

The findings indicated that motility, viability, and MPU values in the control and treatment groups declined over a 5-day storage period at 4 °C. The incorporation of melatonin can preserve the quality of spermatozoa. Melatonin functions as an antioxidant by neutralizing free radicals due to its amphiphilic and lipophilic properties. Melatonin's amphiphilic nature is defined by its capacity to traverse cellular barriers. Conversely, its lipophilic nature is defined by its capacity to traverse the cell plasma membrane (Maitra and Hasan, 2016). Melatonin's lipophilic properties influence the Ca-calmodulin signaling pathway by directly binding to calmodulin and regulating antioxidant enzymes via nuclear receptors in the nucleus (Zaja et al., 2020). The amphiphilic properties of melatonin facilitate its entry into the mitochondria, thereby stabilizing these organelles, as ROS generated by spermatozoa originate from mitochondrial metabolism. Melatonin is a good solution because it can enter and perform its function in the mitochondria to prevent the formation of oxidative stress due to ROS. Fang et al. (2020) reported that melatonin protects the mitochondrial structure of sheep spermatozoa following cryopreservation. Lanconi et al. (2018) indicate that melatonin can enhance the production of antioxidant enzymes, including glutathione peroxidase (GPX) and superoxide dismutase (SOD). Melatonin has receptors called MT1 and MT2, which stimulate the antioxidant effect of melatonin to maintain sperm quality. Melatonin is used as an antioxidant to eliminate ROS levels in spermatozoa, reduce MDA levels, which are the end product of free radicals, and reduce oxidative stress (ChaithraShree *et al*., 2020).

The effort to improve sperm quality through melatonin supplementation is significantly dependent on the administered concentration. According to Mitjana et al. (2022), excessive administration of melatonin to spermatozoa negatively impacts their survival. According to Rodriguez et al. (2020), increased melatonin concentration administered correlates with decreased sperm fertility potential. Kumar et al. (2021) reinforce this point, indicating that administering excessive concentrations of antioxidants may harm spermatozoa by altering the physiological conditions of the diluent. Excessive doses of melatonin may lead to acrosome damage due to increased membrane fluidity (Ashrafi et al., 2011). Based on the research findings, it can be concluded that melatonin preserves sperm motility and viability more effectively than other groups when spermatozoa are stored in tris-fructose egg yolk diluent.

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