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The Effect of Adding Various Concentrations of Melatonin to Beltsville Thawing Solution Diluent on Berkshire Boar Semen Quality

Putu Risma Oktaviandari[1]*, Wayan Bebas[2], Tjok Gde Oka Pemayun[2], Ni Nyoman Werdi Susari[3], and Desak Nyoman Dewi Indira Laksmi[2]

[1]*Master of Veterinary Medicine Student, Faculty of Veterinary Medicine, Udayana University, Bali 80225, Indonesia*
[2]*Department of Reproduction, Faculty of Veterinary Medicine, Udayana University, Bali 80225, Indonesia*
[3]*Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Udayana University, Bali 80225, Indonesia*

*Corresponding author's Email: rismaoktaviandari88@gmail.com

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ABSTRACT

Boar spermatozoa contain polyunsaturated fatty acids, rendering them susceptible to damage from free radicals. Oxidative stress in liquid semen can be prevented by modifying the diluent by adding antioxidants. Melatonin is an indoleamine compound that can be used as an antioxidant with high potential in the reproductive system. This study aimed to determine the effect of adding various concentrations of melatonin to Beltsville Thawing Solution (BTS) diluent on the quality of Berkshire boar semen. A Completely Randomized Design (CRD) method was employed, with the experiment divided into four treatment groups, each replicated six times. A total of 24 samples were used in this study. P0 was used as the control group, while groups P1, P2, and P3 were given the addition of melatonin to BTS diluent with different doses, namely 0.5 mM, 1.0 mM, and 1.5 mM. The samples were stored for 48 hours and then examined for boar semen quality. The variables examined included the percentage of motility, abnormality, viability, plasma membrane integrity, and malondialdehyde (MDA) levels. The results showed that the addition of a melatonin dose of 1.0 mM to the BTS diluent was the optimal concentration that could maintain motility, abnormalities, viability, plasma membrane integrity, and MDA levels in Berkshire boar semen compared to other treatment groups and the control group. This study indicated that melatonin functions as an effective antioxidant, neutralizing free radicals and thereby inhibiting oxidative stress in Berkshire boar semen.

Keywords: Beltsville thawing solution, Berkshire boar, Malondialdehyde, Melatonin, Semen quality

INTRODUCTION

Pigs (*Sus scrofa*) are a significant commodity in the livestock sector, having been farmed for a long time and continuing to hold potential for further development. Pigs have several advantages, such as a fast growth rate (Sarajar et al., 2019), are included in polytocus animals (giving birth to many) with the ability to farrow twice a year or even five times in two years (Armini et al., 2019), and a high feed conversion ratio, yielding a carcass percentage of 65-80% (Wijaya et al., 2019). Giarda and Nugrahini (2020) explained that pork, a major livestock product, is in high demand globally, with worldwide consumption reaching 119,845,000 tons in 2018. According to Susana et al. (2014), as much as 10% of the meat requirements are provided by pigs. In addition, the high demand for pork as a means of traditional and religious customs has also resulted in an increasing need for pork in society.

To fulfill the growing demand for pork, it is essential to increase the pig population. To achieve the goal of increasing the genetic population of pigs, artificial insemination (AI) technology can be utilized through the provision of spermatozoa sources originating from superior-quality males (Sumardani et al., 2008). In addition to other factors, such as the skills of the inseminator, the main success rate in the utilization of AI is influenced by the quality of the boar semen. The processes of dilution and storage are crucial for obtaining the best boar semen quality. At the dilution stage, it is necessary to observe the diluent use. Bebas et al. (2016) mentioned several requirements for semen diluent, including the provision of nutrients as a source of energy for spermatozoa, prevention of cold shock, containing substances that can stop or inhibit the activity of bacteria in semen, acting as a buffer to prevent changes in pH, and maintaining a balance of osmotic and electrolyte pressure. These requirements are critical because fresh semen cannot remain viable for more than 24 hours without proper treatment. Beltsville Thawing Solution (BTS) is one of the diluents currently used for boar semen. According to Foeh et al. (2016), BTS is a diluent with a short shelf life of 1 to 3 days. Nahak et al. (2022) reported that BTS can maintain motility, viability, spermatozoa abnormalities, and pH of boar semen for up to two days of storage. Similarly, Thema et al. (2022) found that BTS preserves the viability, morphology, and motility of boar spermatozoa for 48 hours at 18°C. However, during storage, spermatozoa undergo metabolic processes that not only produce energy but also generate free radicals, which can damage the spermatozoa membrane through lipid

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peroxidation reactions (Leyn et al., 2021). The produced free radicals gradually cause a decrease in semen quality. This is because the plasma membrane of boar spermatozoa has a high level of Polyunsaturated Fatty Acids (PUFA) in phospholipids, making spermatozoa vulnerable to the effect of free radicals (Gena et al., 2021). Bebas et al. (2016) explained that the formation of ROS can cause spermatozoa to experience abnormalities and death. The formation of free radicals also causes an increase in Malondialdehyde (MDA) levels, which is the end product of lipid peroxidation that serves as an indicator of oxidative stress (Gena et al., 2021).

Sperm damage during storage caused by free radicals can be prevented by modifying the diluent solution by adding antioxidant compounds. Recently, there have been many efforts to use antioxidants as additional ingredients in semen diluents to maintain spermatozoa quality (Chankitisakul, 2014). Melatonin (N-acetyl-5-methoxytryptamin) is a compound emerging as an antioxidant. According to Tarocco et al. (2019), melatonin is a molecule that is widely found in nature with several functions, showing extraordinary versatility and diversity. As an antioxidant, melatonin has good potential in improving the reproductive system, particularly in enhancing sperm quality. Its antioxidant properties are manifested either directly through the neutralization of free radicals or indirectly by inducing the activity and expression of major antioxidant enzymes, such as glutathione peroxidase, catalase, and superoxide dismutase (Lavrentiadou et al., 2023).

Studies on melatonin supplementation as an antioxidant have been conducted on spermatozoa in various species, including deer, sheep, horses, buffaloes, and cattle (Chankitisakul, 2014; Li et al., 2019). However, data regarding its effects on boar spermatozoa remains limited. Therefore, this study was conducted to determine the effect of adding melatonin compounds at various concentrations to BTS diluent on the quality of Berkshire boar semen.

MATERIALS AND METHODS

Ethical approval

The Berkshire boars used in this study were handled by workers with expertise in animal care in their fields. This study received ethical permission from the Animal Testing Ethics Committee of the Faculty of Veterinary Medicine, Udayana University, Indonesia, under the reference number B/36/UN14.2.9/PT.01.04/2025.

Study design

This study did not involve the direct use of animals as research objects. The Berkshire boar semen used was obtained from the Regional Artificial Insemination Center in Baturiti, Tabanan, Bali, Indonesia. Semen collection was performed by competent officers in their fields using the hand-glove massage technique. Observations of semen quality were carried out at the Reproduction Laboratory, Faculty of Veterinary Medicine, Udayana University, Denpasar, Indonesia. Malondialdehyde (MDA) level analyses were carried out at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University, Denpasar, Indonesia. In this study, melatonin was added to the BTS diluent at varting doses for each treatment group. The semen was then diluted with the diluent solution to which melatonin had been added at various doses. The semen that had been treated was then stored in the refrigerator at a temperature of 15-20°C for 48 hours. Observations of semen quality consisted of motility, abnormalities, viability, plasma membrane integrity, and MDA levels.

The materials used included Berkshire boar semen, Beltsville Thawing Solution (BTS®) diluent (Minitube, Germany), crystal melatonin (Sigma-Aldrich, USA), eosin-negrosin, Hypo Osmotic Swelling (HOS) solution, aquabides, ethanol, and physiological NaCl. The equipment used included a semen container, a micropipette, a thermometer, an Erlenmeyer flask, a Microscope Binocular Olympic (Olympus Corporation, Japan), a 10 ml plain vaculab tube, a pair of object glass, a pair of cover glass, a Porcine Malondialdehyde Enzyme-Linked Immunosorbent Assay (ELISA) kit No. E0151Po (BT LAB, China), a cool box, a 2 ml eppendorf tube, ice cubes, a water bath, a pair of hand gloves, a mask, an incubator, a magnetic stirrer, a centrifuge, and an ELISA microplate reader. This experimental study employed a completely randomized design. The samples were divided into four treatment groups and six replications in each group. The treatment groups were as follows: P0 (Control group, without melatonin), P1 (BTS + Melatonin 0.5 mM), P2 (BTS + Melatonin 1.0 mM), and P3 (BTS + Melatonin 1.5 mM).

Diluent preparation

Crystalline Melatonin M5250 (Sigma-Aldrich, USA) was added to the BTS diluent. To make the diluent usable, calculations were made to determine the melatonin preparation and the volume of BTS required using the molarity formula. From the calculation results, it became clear that mixing 1 ml of melatonin and 215 ml of BTS led to a BTS diluent with a melatonin concentration of 1 mm. The preparation of other concentrations followed the above calculations. Each diluent was homogenized using a magnetic stirrer and incubated at 37°C.

Evaluation of sperm motility

Spermatozoa motility was assessed by placing one drop of semen on a glass slide and examining it under a binocular microscope (Olympus Corporation, Japan) at 400x magnification. A minimum of 5-10 fields of view were observed for evaluation (Sumardani et al., 2019).

Evaluation of sperm abnormality and viability

Sperm abnormalities and viability were evaluated by making a smear preparation using eosin-nigrosin dye. Evaluation of spermatozoa abnormalities was based on morphological abnormalities that occur in the head to tail of the spermatozoa. In the evaluation of viability, live and dead spermatozoa were distinguished based on their color absorption ability. Dead spermatozoa appeared red, while live spermatozoa remained unstained/transparent (Baku et al., 2022). The evaluation was carried out using a microscope with a magnification of 400×. Evaluation of spermatozoa abnormality was counted in a minimum of 10 fields of view with the number of all normal and abnormal spermatozoa counted as 200 cells (Butta et al., 2021).

Evaluation of plasma membrane integrity

Plasma membrane integrity was evaluated using the Hypo Osmotic Swelling Test (HOST) method. A total of 20 ml of hypoosmotic solution was added to 0.2 ml of semen, mixed until homogeneous, and then incubated at 37°C for 45 minutes (Bebas dan Agustina, 2022). After incubation, 0.2 µL of the solution was dropped onto a glass for further observation using a microscope with a magnification of 400×. The number of spermatozoa was observed in at least 200 cells in 10 fields of view (Nofa et al., 2017). Spermatozoa with intact plasma membranes exhibited circular or bulging tails, while those with damaged membranes had straight tails.

Evaluation of malondialdehyde level

A Malondialdehyde level test was conducted to measure the degree of oxidative stress in spermatozoa after treatment. The ELISA kit used in the study was Porcine Malondialdehyde ELISA kit No. E0151Po (BT LAB, China), is a special kit for detecting MDA in various tissues, serum, plasma, and other biological fluids in pigs. The ELISA test is based on the binding of antigens in the sample to antibodies in the kit wells. The research procedure was carried out in accordance with the ELISA Kit's user manual.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) in IBM SPSS Statistics version 26. If significant differences ($p < 0.05$) were detected, post-hoc analysis was performed using Duncan's Test.

RESULTS AND DISCUSSION

The macroscopic evaluation showed that the collected volume of fresh Berkshire boar semen was 280 ml and had a milky white color, watery consistency, and typical odor. Based on microscopic results, the fresh semen had 78% motility, 90% viability, and 4% abnormality. Evaluation of fresh semen quality is essential in determining the quality of spermatozoa produced by each animal (Komariah et al., 2020). Normally, fresh boar semen has a milky white color with a typical odor. Butta et al. (2021) also noted that boar semen is voluminous, with ejaculate volumes ranging from 100 to 500 ml. Changes in color, such as a pinkish hue, may indicate bleeding or infection in the urinary tract. In addition, Tamoes et al. (2014) explained that qualified fresh semen should have a motility percentage of ≥ 60%, a live spermatozoa percentage of ≥ 70%, and an abnormality rate of ≤ 20%. Based on these observations, the fresh boar semen in this study was of high quality and, therefore, suitable for further processing. The results of the evaluation of Berkshire boar spermatozoa quality after treatment are presented in Table 1.

Table 1. The evaluations of Berkshire boar sperm quality (Mean ± SD) diluted with BTS after adding various concentrations of melatonin

Result / Components	P0	P1	P2	P3
Motility (%)	40.83 ± 0.98^{a}	45.16 ± 1.32^{b}	49.33 ± 0.81^{c}	40.00 ± 0.63^{a}
Abnormality (%)	7.16 ± 0.75^{b}	$6.83 \pm 0.98^{a,b}$	6.00 ± 0.89^{a}	7.33 ± 1.03^{b}
Viability (%)	49.67 ± 1.50^{ab}	51.00 ± 0.89^{a}	61.00 ± 1.26^{c}	49.33 ± 0.81^{b}
Plasma Membrane Integrity (%)	50.33 ± 0.81^{a}	55.50 ± 1.04^{b}	62.16 ± 0.75^{c}	49.33 ± 0.81^{a}
MDA Levels (ng/ml)	1979.39 ± 108.76^{b}	1997.13 ± 134.03^{b}	1792.11 ± 106.42^{a}	2050.53 ± 147.34^{b}

[abc] Different superscript letters in the same rows indicate significantly different results ($p < 0.05$). P0: Control Group; BTS Only; P1: BTS + Melatonin 0.5 mM; P2: BTS + Melatonin 1.0 mM; P3: BTS + Melatonin 1.5 mM. MDA: Malondialdehyde.

Evaluation of sperm motility

The motility, or progressive movement, of spermatozoa is the simplest assessment in determining semen quality. Spermatozoa motility is very important because it should move forward in the female reproductive tract and fertilize the ovum (Baku et al., 2022). The movement of spermatozoa is facilitated by flagella, with the main driving force being axonema, which is formed by microtubules derived from centrioles in the cell nucleus. The progressive movement or motility of spermatozoa is caused by friction between microtubules due to the presence of oxygen derived from dynein (Gazali and Tambing, 2002). At this stage, the availability of oxygen is required to convert chemical energy into mechanical energy. In addition, Solihati et al. (2020) explained that the processing of fresh semen into liquid semen allows a lot of contact between semen and the outside air, which contains reactive oxygen species (ROS). Semen containing excessive ROS levels will cause accelerated metabolism and end in the formation of free radicals. The continuous formation of free radicals' results in lipid peroxidation reactions that are autocatalytic and very difficult to stop. In this case, antioxidant compounds can suppress or prevent oxidative stress by neutralizing free radicals (Feradis, 2009). In this study, the results showed significant differences ($p < 0.05$) across most groups. In groups P0 and P3, however, there was no significant difference ($p > 0.05$). The P2 treatment group had the highest average percentage of motility compared to the other groups, amounting to 49.33 ± 0.81%. The lowest average percentage of spermatozoa motility was obtained in the P0 control group, reaching 40.83 ± 0.98%. Nevertheless, the addition of excessive antioxidant compounds can also have adverse effects since compounds that are given in excess will cause toxicity, so compounds that were originally antioxidants turn into prooxidants. In general, high dosages of antioxidants can act as prooxidants, bind with specific molecules to generate free radicals, or do so because of other reasons. Under certain conditions, melatonin can increase singlet oxygen, peroxy radicals, and hydroxyl radicals (Sotler et al., 2019). However, no study has yet explained in detail the role of melatonin as a prooxidant in semen quality. Karepu et al. (2020) stated that a prooxidant is a compound that can encourage the oxidation of cell components involving free radical compounds. This theory is consistent with the results of the motility observation of group P3, which was treated by adding 1.5 mM melatonin to the BTS diluent. In this group, the average percentage of motility obtained was very low, which amounted to 40.00 ± 0.63%. This result was at the threshold of the Indonesian National Standard (SNI 8030: 2014), which stated that the minimum percentage of semen motility that was suitable for use in artificial insemination was 40% (Parera et al., 2018).

Evaluation of sperm abnormality

Abnormality is a state of morphological deviation in spermatozoa cells. According to Parera and Lenda (2023), spermatozoa abnormality is an indication of decreased fertility because it reduces spermatozoa capacitation at the time of fertilization and affects the development and maintenance of pregnancy. Abnormality of spermatozoa is one of the important factors in determining the quality of spermatozoa; if the percentage of abnormality is above 20% in the semen used in artificial insemination, the fertility rate will be low due to the absence of fertilization during copulation (Prastiwi et al., 2021). In this study, the highest average abnormality percentages were observed in the P0 and P3 groups ($p < 0.05$). The high percentage indicated that there was an increase in morphological deviation of spermatozoa from the percentage of abnormalities in fresh semen examination of 4%. The increase in abnormality in group P0 (Control Group) was attributed to the absence of antioxidants in the diluent, while in P3, it resulted from melatonin toxicity due to excessive concentration in the diluent. Both treatment groups caused the semen to experience oxidative stress due to increased formation of lipid peroxidation. This finding is in line with Amtiran et al. (2020), who explained that the increase in abnormality rate was not only caused during the preparations before observation but also caused by lipid peroxidation. Lipid peroxidation can cause damage to the structure and metabolism of spermatozoa which, in turn, will cause increased damage to spermatozoa morphology (Wang et al., 2025). Medrano et al. (2017) further noted that oxidative stress impairs spermatozoa morphology and function due to the formation of excessive free radicals. The results of the abnormality evaluation are presented in Figure 1A.

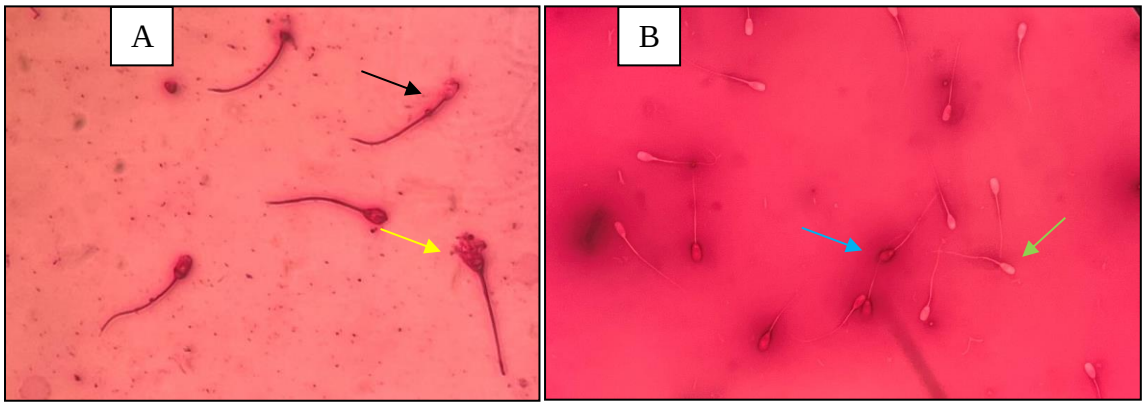

Figure 1. The abnormality (A) and viability (B) of Berkshire boar spermatozoa with eosin-negrosin staining at 400× magnification. **A**: Abnormality in spermatozoa with spermatozoa with abnormalities, such as cytoplasmic droplets (black arrow); abnormalities on the head of spermatozoa (yellow arrow). **B**: Dead spermatozoa absorb color (blue arrow); live spermatozoa do not absorb color (green arrow).

Evaluation of sperm viability

Under normal physiological conditions, stored spermatozoa, after being treated with dilution, metabolize to maintain their life. The main metabolic processes in spermatozoa consist of glycolysis and respiration. During glycolysis, spermatozoa utilize carbohydrates present in the diluent as nutrients to generate ATP. The glycolysis process can occur through two pathways, namely, metabolism with the fructose breakdown pathway and the glucose breakdown pathway. In the respiration process, spermatozoa utilize various substrates as a source of oxygen (Susilawati, 2011). Furthermore, Susilawati (2011) explained that spermatozoa respiration uses lactic acid and pyruvate derived from fructose breakdown to produce CO_2 and water. Metabolism that occurs continuously causes a reduction in available nutrients, resulting in a gradual decrease in spermatozoa viability. In addition, the metabolic process in spermatozoa cells causes a reaction between spermatozoa and oxygen, leading to the production of free radicals or ROS. Rizal and Herdis (2010) noted that while excess O_2 causes peroxidative damage, ROS compounds are formed from cell metabolism activities during semen processing during collection, dilution, and storage (Bebas et al., 2016). An imbalance between free radical formation and antioxidant levels within the cell (Sholihin and Ducha, 2024), further exacerbated by the absence of antioxidants in the diluent (Nalley et al., 2024), can induce oxidative stress, ultimately affecting the survival of spermatozoa. Lawa et al. (2021) explained that spermatozoa cell death, also known as apoptosis, can occur in the absence of antioxidant protection. This finding aligns with the results of the present study, which demonstrated a significant decline in sperm viability in the P0 group, where spermatozoa were diluted using BTS diluent without the addition of melatonin. Duncan's statistical test revealed significant differences ($p < 0.05$) in the evaluation of the viability of spermatozoa in this study. The highest viability percentage was obtained from the P2 treatment group, which amounted to 61.00 ± 1.26%. These results indicated that the addition of melatonin as an antioxidant compound in the diluent can maintain the balance of antioxidants and free radicals so that the decrease in viability percentage occurred more slowly as compared to other treatment groups. The results of the viability evaluation are presented in Figure 1B.

Evaluation of plasma membrane integrity

The plasma membrane plays an important role in spermatozoa, serving as the primary defense against external environmental damage to the cells. The functions of the plasma membrane are not limited only to protecting the organelles contained in the cell; it can function as a potential filter for the exchange of intra- and extracellular substances (Bahmid et al., 2023). Boar spermatozoa are unique in their structure, containing higher unsaturated fatty acids when compared to other mammals. Khophloiklang et al. (2024) explained that the plasma membrane of boar spermatozoa contains polyunsaturated fatty acids, such as Docosapentaenoic Acid (DPA) and Docosahexaenoic Acid (DHA). The phospholipid structure of the spermatozoa plasma membrane, which contains high unsaturated fatty acids, causes spermatozoa cells to be vulnerable to free radicals and trigger autocatalytic reactions, leading to the breakdown of their double bonds (Tamoes et al., 2014).

Figure 2. Evaluation of plasma membrane integrity of Berkshire boar spermatozoa after treatment with Hypo Osmotic Swelling Test (HOST). 400× magnification; normal plasma membrane integrity is characterized by a swelling tail (black arrow).

Duncan's statistical test showed that there were significant differences ($p < 0.05$) in the evaluation of plasma membrane integrity. The highest percentage of plasma membrane integrity was obtained in group P2, followed by group P1 with a percentage of plasma membrane integrity of 55.50 ± 1.04%. These results indicated that the addition of melatonin to BTS diluent can act as an antioxidant to maintain the integrity of the plasma membrane against the damage caused by free radicals. High levels of ROS lead to oxidative stress, which can affect cell metabolism. This is because free radicals damage the plasma membrane of spermatozoa, which will, in turn, cause damage to organelles in spermatozoa cells, where Adenosine Triphosphate (ATP) is produced during cellular respiration (Priharyanthi et al., 2021). Ultimately, decreased or damaged plasma membrane integrity results in reduced sperm motility, increased morphological abnormalities, and cell apoptosis. The results of the viability evaluation are presented in Figure 2.

The results from groups P0 and P3 showed no significant difference ($p > 0.05$), with both groups exhibiting the lowest plasma membrane integrity compared to groups P1 and P2. In group P0, the absence of antioxidant compounds in

the BTS diluent led to the absence of compounds capable of balancing free radicals. In group P3, excessive antioxidant dosage converted antioxidants into prooxidants, inducing toxicity. Plasma membrane integrity plays a critical role in spermatozoa motility. Setiadi et al. (2006) stated that if the plasma membrane of spermatozoa is damaged, the metabolism of spermatozoa will be disrupted, leading to loss of motility and, ultimately, the death of spermatozoa.

Malondialdehyde levels

Malondialdehyde (MDA) level testing is conducted to determine the level of oxidative stress in semen. This test measures the end product of free radicals, as direct measurement of free radicals is not feasible due to their autocatalytic nature and short half-life, which causes their disappearance within a few seconds (Hayati et al., 2006). MDA formation occurs through both enzymatic and nonenzymatic processes (Cordiano et al., 2023; Rizzo et al., 2024). In the formation through enzymatic processes, MDA is formed as a by-product during the formation of Thromboxan A2 (TXA2) whereas in the non-enzymatic processes, MDA is formed as a secondary product of lipid peroxidation, which is often used to measure the degree of oxidative stress (Bikulčienė et al., 2019). According to Hayati et al. (2006), MDA is a compound that is the result of lipid oxidation into peroxides through a series of processes called lipid peroxidation. The term lipid peroxidation is generally defined as a process of oxidative lipid degradation. According to Ayala et al. (2014), lipid peroxidation is the process of reaction between oxidants and lipids containing oxidant carbon-carbon double bonds, such as polyunsaturated fatty acids (PUFAs). This reaction involves the removal of hydrogen from carbon with oxygen insertion, resulting in the formation of lipid peroxyl radicals and hydroperoxides.

In the study, the result of Duncan's test showed significant differences in MDA levels among the groups ($p < 0.05$). The highest MDA levels were obtained in the P3, P1, and P0 groups, respectively, with MDA levels of 2050.53 ± 147.34 ng/ml, 1997.13 ± 134.03 ng/ml, and 1979.39 ± 108.76 ng/ml. The MDA level in group P0 was high because the diluent in this group did not contain antioxidants, while in group P1, even though melatonin had been added at a dose of 0.5 mM, it could not act as an antioxidant compound to convert reactive oxygen into neutral compounds. In group P3, high MDA levels were caused by the high dose of the administered melatonin, which caused toxicity. In this case, instead of acting as an antioxidant, melatonin transitioned into a prooxidant, accelerating oxidative stress. Munik and Ekmekçioğlu (2015) reported that under certain conditions, antioxidants can exhibit prooxidant behavior. However, the stages or processes of melatonin acting as a prooxidant have not been explained in detail, with most of the literature describing the prooxidant effects of melatonin as a hormone in the body rather than a supplement. Vega-Naredo et al. (2005) emphasized that the prooxidant effects of melatonin have only been reported *in vitro* cell culture systems, especially on cancer cells, and cannot, therefore be generalized to other cells and systems.

Oxidative stress in spermatozoa causes disturbances in the phosphorylation oxidation process, resulting in increased production of spermatozoa ROS (Hayati et al., 2006). On the other hand, the cell structure of spermatozoa in the cell membrane, which contains a lot of unsaturated polyunsaturated fatty acids, causes faster oxidative stress. Situmorang and Zulham (2020) explained that the double bonds of unsaturated fatty acids are the target of free radicals, triggering a cascade of fatty acid degradation in their vicinity. The test results showed that the lowest MDA levels were obtained in group P2, indicating that the administration of melatonin at a dose of 1.0 mM exhibited the most effective antioxidant properties in preventing oxidative stress compared to other groups. Garcia et al. (2014) highlighted the potent antioxidant properties of melatonin, emphasizing its ability to mitigate oxidative stress through multiple mechanisms. In the context of lipid peroxidation, melatonin can react with ROS, such as hydroxyl radicals. In addition, the result of the metabolism that occurs between melatonin and ROS can also neutralize free radicals. According to Zhang and Zhang (2014), melatonin detoxifies ROS through a sequential process that results in the production of cyclic 3-hydroxy melatonin (3-OHM/c3OHM), a compound that also functions as a ROS scavenger. When 3-OHM reacts with ROS, it undergoes oxidation, resulting in the formation of N^1-acetyl-N^2-formyl-methoxykynuramine (AFMK). In its stages, AFMK will then undergo deformylation, which causes the formation of N^2-acetyl-5-methoxynuramine (AMK). Reiter et al. (2014) further explained that both AFMK and AMK serve as effective peroxyl radical scavengers, reinforcing the role of melatonin in mitigating oxidative stress.

CONCLUSION

The findings of this study indicated that the administration of melatonin at a dose of 1.0 mM in Beltsville Thawing Solution (BTS) diluent is the most effective dosage for preserving the viability of Berkshire boar liquid semen. Based on these results, it is recommended that future research be conducted through *in vivo* testing in sows through the stages of artificial insemination to find out whether liquid semen supplemented with melatonin in the diluent can increase the number of piglets born or not.

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Availability of data and materials
The data of the current study are available upon reasonable request from the corresponding author.

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Authors' contributions
Putu Risma Oktaviandari wrote the manuscript and conducted the research, and Wayan Bebas and Tjok Gde Oka Pemayun supervised the research and revised the final manuscript. Ni Nyoman Werdi Susari and Desak Nyoman Dewi Indira Laksmi provided tips and feedback on the manuscript. All authors reviewed and approved the final edition of the manuscript.

Competing interests
The authors declare that they have no competing interests.

Ethical considerations
The authors confirmed that the manuscript has been submitted for the first time to this journal. All ethical issues, including plagiarism, consent to publish, misconduct, data fabrication, double publication, and redundancy, have been checked by all the authors.

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