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QUALITY OF BERKSHIRE BOAR SEMEN DILUTED WITH A COMBINATION OF BELTSVILLE THAWING SOLUTION AND MELATONIN AT DIFFERENT STORAGE TIMES

Kualitas Semen Babi *Berkshire* yang Diencerkan dengan Kombinasi *Beltsville Thawing Solution* dan Melatonin pada Waktu Simpan yang Berbeda

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Abstract

Berkshire pigs are a popular choice for small and large scale pig farming enterprises worldwide, including in Bali. Berkshire pigs are well known for their superior meat quality and relatively fast growth rate. This study aimed to determine the effect of storage duration on the quality of Berkshire boar spermatozoa diluted with Beltsville Thawing Solution (BTS®) supplemented with melatonin. Semen from Berkshire boars was diluted using BTS® supplemented with melatonin at a concentration of 1.0 mM and stored at a temperature of 15 –20°C. Semen quality was evaluated at different storage intervals: (P1) 48 hours, (P2) 60 hours, and (P3) 72 hours. The observed semen quality parameters included sperm motility, sperm abnormalities, sperm viability, and plasma membrane integrity of spermatozoa, which were examined using smear preparations observed under a microscope. In addition, sperm malondialdehyde (MDA) levels as an indicator of oxidative stress were measured using the Porcine MDA ELISA Kit. Duncan's multiple range test analysis showed that the storage duration of Berkshire boar semen diluted with BTS® supplemented with melatonin was able to maintain sperm motility, abnormality, viability, plasma membrane integrity, and malondialdehyde levels up to 72 hours of storage, with sperm motility of 40.66%, sperm viability of 53.00%, and sperm abnormalities of 6.33%. These values met the minimum standards set by the Indonesian National Standard (SNI

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8034:2023) for liquid boar semen. These results indicate that melatonin acts as an antioxidant capable of neutralizing free radicals, thereby inhibiting the occurrence of oxidative stress in spermatozoa cells.

Keywords: Berkshire Boar, Motility, Abnormality, Viability, Plasma Membrane Integrity, Malondialdehyde

Abstrak

Babi *Berkshire* menjadi pilihan populer bagi perusahaan peternakan babi skala kecil dan besar di seluruh dunia termasuk di Bali. Babi *Berkshire* dikenal sebagai salah satu bangsa babi dengan kualitas daging yang unggul serta laju pertumbuhan yang relatif cepat. Penelitian ini bertujuan untuk mengetahui pengaruh lama waktu simpan spermatozoa babi *Berkshire* yang diencerkan dengan *Beltsville Thawing Solution* (BTS®) yang ditambahkan melatonin terhadap kualitas semen. Semen babi Berkshire jantan diencerkan menggunakan BTS®) yang ditambahkan melatonin dengan konsentrasi 1,0 mM, kemudian disimpan pada suhu 15–20°C. Pengamatan kualitas semen dilakukan pada interval waktu simpan (P1) 48 jam, (P2) 60 jam dan (P3) 72 jam. Parameter kualitas semen yang diamati meliputi motilitas spermatozoa, abnormalitas spermatozoa, viabilitas spermatozoa, integritas membran plasma utuh spermatozoa metode pemeriksaan dengan membuat preparat ulas diamati dibawah mikroskop. Kemudian pemeriksaan kadar *Malondialdehyde* (MDA) spermatozoa sebagai indikator stres oksidatif menggunakan uji *Porcine* MDA ELISA Kit. Dari hasil analisis uji *Duncan* menunjukkan bahwa lama waktu simpan semen babi Berkshire yang diencerkan dengan BTS® dengan penambahan Melatonin terhadap kualitas semen dapat mempertahankan motilitas, abnormalitas, viabilitas, integritas membran plasma, dan kadar MDA semen babi Berkshire hingga lama waktu penyimpanan 72 jam dengan nilai persentase motilitas spermatozoa 40,66%, viabilitas spermatozoa 53,00%, abnormalitas spermatozoa 6,33% hasil ini berada pada nilai minimum (SNI 8034:2023) Semen Cair Babi. Hal ini menunjukkan bahwa melatonin mampu berperan sebagai antioksidan yang dapat menetralkan radikal bebas sehingga dapat menghambat terjadinya stres oksidatif pada sel spermatozoa.

Kata Kunci: Babi Berkshire, Motilitas, Abnormalitas, Viabilitas, Integritas Membran Plasma, *Malondialdehyde*

INTRODUCTION

Pigs are one of the livestock commodities that produce meat and have great potential for development because they have beneficial characteristics and abilities, including fast growth rates, high litter sizes, and high carcass percentages (65-80%) (Nahak *et al.*, 2022). Berkshire pigs are a popular choice for small and large pig farms around the world, including in Bali. In addition to being a source of animal protein, pigs also have strong social and cultural value in Balinese society. Berkshire pigs are known as one of the pig breeds with superior meat quality and relatively fast growth rates. Another advantage of Berkshire pigs is their black body color, which makes them particularly attractive to the Balinese people. This is due to the high demand for black pigs in the performance of various religious ceremonies.

The extensive use of pigs in Balinese society has led to high demand for pork, requiring a balance between pork demand and pig population. Livestock production can be increased by optimizing reproductive efficiency, one of which is through artificial insemination (AI) using semen from superior boars (Sumardani *et al.*, 2019). AI in pigs in Indonesia still uses liquid semen, where the processing of liquid semen must take into account various aspects such as the composition of diluents and semen storage (Johnson *et al.*, 2000).

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In this study, the diluent used was Beltsville Thawing Solution, which is a diluent with a short shelf life (Zhou *et al.*, 2004). In its use in the field, Beltsville Thawing Solution can only maintain sperm motility suitable for AI for approximately 48 hours (Nalley *et al.*, 2024). The composition of the diluent includes *Ethylene Diamine Tetraacetic Acid* (EDTA), which plays a role in protecting the plasma membrane, and glucose, which provides nutrients for spermatozoa. there is also sodium bicarbonate and sodium citrate which act as buffers that can maintain pH stability for the survival of spermatozoa, antibiotics (penicillin, streptomycin) which play a role in suppressing bacterial growth, and aquabidest which plays a role in diluting semen (Dube *et al.*, 2004).

Another obstacle that often arises in the production of liquid pig semen is the storage process. Mammalian spermatozoa are rich in unsaturated fatty acids and are easily affected by reactive oxygen species (ROS) groups, which can reduce motility and increase morphological damage to spermatozoa (Situmorang and Zulham 2020). Sperm cell damage can be measured through *Malondialdehyde* (MDA) levels. The MDA is a dialdehyde compound that is the end product of lipid peroxidation that occurs in lipid membranes (Mahfouz *et al.*, 2012). Free radicals can increase lipid peroxidation, which then decomposes into MDA. The MDA test can be used to measure peroxidation occurring in the lipid membrane; the higher the radical level, the higher the MDA level formed (Mahfouz *et al.*, 2012).

To counteract free radical attacks during semen storage, antioxidants need to be added to the diluent. The addition of antioxidants is expected to inhibit lipid peroxidation reactions, as antioxidants are substances that can bind free radical compounds. One antioxidant that can be used is melatonin. Melatonin is one of the most effective antioxidants that protects cells from oxidative stress caused by reactive species. The lipophilic nature of melatonin allows it to easily cross cell membranes and work directly in various organs, including the reproductive system (Jang *et al.*, 2014).

Recent studies evaluating the effects of melatonin on sperm cryopreservation have focused on cattle (Ofosu *et al.*, 2021), goats, rams, and buffalo (Rateb *et al.*, 2020; Inyawilert *et al.*, 2021; Pool *et al.*, 2020) has been shown to protect sperm from oxidative damage, maintain sperm viability, reduce morphological abnormalities, and prevent DNA fragmentation (Gualtieri *et al.*, 2021). The National Standardization Agency has established SNI 8034: 2023 Liquid Pig Semen in accordance with BSN Head Decree Number 512/KEP/BSN/11/2023, with the final result of the Indonesian National Standard (SNI) requiring a minimum sperm motility of 40% before insemination, a maximum abnormality rate of 20%, a minimum individual spermatozoa movement score of 2 (two), and straight-line sperm movement. However, research and literature on the effect of melatonin supplementation on boar semen quality are still very limited, so further research is needed. This study was conducted to determine the effect of storage time on the quality of Berkshire boar semen in Beltsville Thawing Solution diluted with melatonin.

RESEARCH METHODS

Ethical Feasibility of Experimental Animals

This study does not require ethical feasibility because the samples used were in the form of fresh semen *of* Berkshire pigs collected using the method of massage on the penile glands, and the animals sampled did not receive treatment.

Research Object

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The object of the study was a male Berkshire pig, aged 3 years with a weight of between 150-250 kg. The pigs were selected based on the criteria of healthy body condition, high libido and normal reproductive organs. In addition, the pig has been trained as a cement producer.

Research Design

This research is a laboratory experimental research with a Complete Random Design (RAL) research design. The pig semen obtained was then divided into three groups, namely P1 is pig semen with BTS® + Melatonin diluent 1.0 mM with a shelf life of 48 hours, P2 is pig semen with BTS® + Melatonin diluent 1.0 mM with a shelf life of 60 hours, P3 is a pig semen with BTS® + Melatonin diluent 1.0 mM with a shelf life of 72 hours. The diluted cement is stored at a temperature of 15-18^{o}C, then observations are made on the quality of the cement in multiples of 12 hours as a factor for 72 hours. The number of samples was calculated based on the free degree formula popularized by Federer, namely $(t-1)(n-1) \geq 15$.

Research Variables

The variables of this study consist of 1). Independent variable: cement storage time, in this study the quality of Berkshire pig cement was observed at 48 hours, 60 hours, and 72 hours. 2). Bound Variables: spermatozoa motility, spermatozoa abnormality, spermatozoa viability, spermatozoa intact plasma membrane and MDA levels of Berkshire pig spermatozoa 3). Control variables: type of diluent used, age of Berkshire pigs, body weight and motility standards of semen accommodated at least 70%.

Data Collection Methods

Cement Collection

The cement storage process was carried out at the Technical Implementation Unit of the Regional Artificial Insemination Center (UPT BIBD), Livestock Nursery and Forage Feed/Bali Provincial Livestock Institute, Baturiti, Tabanan. The process of collecting pig semen is carried out using the massage method on the penile gland. This technique is applied when the male rides a dummy (artificial female), with the aim of stimulating ejaculation naturally and effectively.

Preparation of Diluting Ingredients

The calculation of the amount of dilution used is based on the cement motility standard used by the UPTD. Regionally Made Insemination Center, Livestock Nursery and Forage Feed/Bali Provincial Livestock Institute, Baturiti, Tabanan. Volume of Capacity X Number Comparison.

Creation of Melatonin Concentration

Solution Solubility/Stability (Sigma-Aldrich): Melatonin is soluble in ethanol at least up to 50 mg/mL. A 250 mg preparation of crystalline Melatonin (Sigma Aldrich, M5250) means it is dissolved in 0.02 ml of ethanol. Melatonin preparation 250 mg = 0.25 g dissolved with Aquabides as much as 5 ml then the Melatonin content per ml = 0.05 g Melatonin. A preparation of 1 ml of Melatonin is added to 215 ml of BTS® so that it becomes a BTS® diluent with a Melatonin concentration of 1 mM.

Cement Quality Evaluation

Fresh cement from the reservoir was examined both macroscopically and microscopically. The evaluation of pig semen included motility, abnormality, viability, MDA levels, and plasma membrane integrity. The evaluation of motility was carried out by dripping one drop of liquid cement on the glass of the object and then closed using a glass cover. The viability and abnormalities of spermatozoa were checked by eosin-nigrosine staining, then 50µL of cement

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with 5µL of eosin-nigrosine was mixed and then homogenized. The smear preparation is made after 30 seconds and dried by aerating. The integrity of the plasma membrane of spermatozoa is generally observed by the Hypo Osmotic Swelling Test (HOST) method. The hypoosmotic solution consists of 0.9 g of fructose + 0.49 g of sodium citrate dissolved in aquades to a volume of 100 mL. Next, 20 mL of the solution was added with 0.2 mL of cement, mixed until homogeneous and incubated at 37°C for 45 minutes (Bebas and Agustina, 2022).

Evaluation of the motility, abnormality, viability and integrity of the plasma membrane was carried out using microscopes observed approximately 200 spermatozoa cells using a microscope with a magnification of 400x. The evaluation of MDA levels was carried out using the ELISA test. The ELISA kit used is the Porcine MDA ELISA kit No. E0151Po (BT LAB, China) which is a special kit for detecting MDA of various tissues, serums, plasma and other biological fluids in pigs.

Data Analysis

Data analysis uses analysis of variance (ANOVA), if there is a real difference ($p<0.05$), then it is followed by a statistical test using the Duncan Test.

RESULTS AND DISCUSSION

Results

Macroscopic assessment can be done by direct observation, which includes a volume of 280 ml, milky white color, thin consistency, distinctive pig odor, and pH of 7.5. In general, the characteristics of fresh semen produced are not much different from the results of other researchers. Garner and Hafez (2000) stated that pig semen is voluminous, with an ejaculate volume of 100-500 ml and a low sperm concentration of 200-300x106 cells/ml, milky white color, thin consistency, and an average pH of 7.40 ±0.2 (Gadea, 2003). Microscopic examination of the fresh semen collected in this study showed rapid mass movement (++) with a motility percentage of 78%, viability of 90%, and abnormality of 4%. These results indicate that the quality of the fresh semen is within the normal range. Mass movement is an assessment of the movement of spermatozoa in groups (Susilawati *et al.*, 2022).

Observation of spermatozoa movement by looking at the tendency to move together in one direction, forming thick, fast-moving waves (+++), thin, fast-moving waves or thick, slow-moving waves (++), thin, slow-moving waves (+), and no movement (0) (Nahak *et al.*, 2022). Criteria for determining semen quality based on individual motility with grades 0-5 (Toelihere, 1993): (0): Immotile or non-moving sperm, (1): Spinning in place, (2): Oscillating or circular movement with less than 50% progressive movement, (3): 50%–80% spermatozoa moving progressively, (4): Agile progressive movement, 90% motile sperm, (5): Highly progressive movement showing 100% active motile spermatozoa.

Apriliani *et al.* (2021) stated that fresh semen suitable for dilution must meet the requirements of viability ≥70%, motility ≥70%, and abnormality ≤20%. Based on the observation results of fresh boar semen in this study, it is suitable for further processing because it already meets the percentage requirements. The results of the evaluation of the quality of Berkshire boar spermatozoa after dilution and addition of 1.0 mM melatonin antioxidant are presented in Table 1.

Discussion

Spermatozoa Motility

Motility or spermatozoa motility plays a very important role in the success of the fertilization process (Susilawati *et al.*, 2003). In a study conducted by Oktaviandari *et al.* (2025) on the

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addition of various concentrations of melatonin to Beltsville Thawing Solution diluent on the quality of Berkshire boar semen, it was found that the treatment group with the addition of 1.0 mM melatonin had the highest average motility percentage compared to the other groups, namely 49.33±0.81%. In this study, observations of Berkshire boar sperm motility were continued up to a storage time of 72 hours.

The analysis of variance results showed that the effect of the storage time treatment in BTS® diluent with the addition of 1.0 mM melatonin had a significant effect ($P<0.05$) on the motility of Berkshire boar sperm. Based on Duncan's post-hoc test, it was found that the highest average motility value was in the P1 treatment group (49.33±0.86%), followed by P2 (45.44±1.33%) and P3 (40.66±1.22%). BTS® diluent with the addition of 1.0 mM melatonin antioxidant was able to maintain sperm motility for up to 72 hours of storage with a sperm motility percentage of 40.66%, This result meets the minimum requirement of the Indonesian National Standard (SNI 8030:2014), which states that the percentage of motile semen suitable for artificial insemination (AI) must be at least 40% (Parera *et al.*, 2023).

The decrease in sperm motility percentage in BTS® diluent also occurs because the nutrient source for sperm begins to decrease. Semen storage continues to decline with the duration of semen storage. Sikka (2004) and Maldjian *et al.* (2005) stated that mammalian spermatozoa are rich in unsaturated fatty acids and are highly susceptible to ROS, which can cause a decrease in sperm motility. The results of this study are still better than those of Zhang *et al.* (2009), who used 6% (w/v) soy lecithin supplementation in BTS® diluent on Duroc pig spermatozoa and were able to maintain a motility percentage of 44% during 48 hours of storage. The results of this study are also better than those of Nalley *et al.* (2024). A comparison of the quality of Landrace pig sperm in Beltsville Thawing Solution, Mulberry III, and Sperm Life diluents supplemented with moringa leaf ethanol extract observed for 48 hours showed that the Beltsville Thawing Solution diluent had a percentage of 44.40±5.5%, Mulberry III 39.60±5.6%, and Sperm Life 27.00±5.7%.

The addition of melatonin antioxidants in this study yielded better results than those obtained by Makleat *et al.* (2024). The results showed that the addition of 0.05% vitamin C in the Beltsville Thawing Solution had a positive effect on maintaining the quality of Landrace pig spermatozoa during 40 hours of storage, which was 55.00±3.53%, but there was a drastic decrease during 48 hours of storage, which was 34.00±2.24%. Meanwhile, in this study, the results of adding the antioxidant melatonin were even better, as it was able to maintain sperm motility for up to 72 hours of storage, with an average of (40.66±1.22%).

Spermatozoa viability

Sperm viability is the ability of spermatozoa to survive after dilution and is one of the important factors in determining the quality of spermatozoa from a male animal. The higher the sperm viability, the higher the chance of fertilization during copulation, both naturally and artificially (Manehat *et al.*, 2021). The analysis of variance results showed that the effect of storage duration in Beltsville Thawing Solution with the addition of 1.0 mM melatonin had a significant effect on the viability of Berkshire pig spermatozoa. Based on Duncan's post-hoc test, it was found that the highest average viability value was in treatment P1 with a storage period of 48 hours (61.00±1.11%), followed by P2 with a storage period of 60 hours (55.55±1.87%) and P3 with a storage period of 72 hours (53.00±1.32%). There were significant differences between treatments ($p<0.05$).

Factors causing a decrease in sperm viability during storage can be attributed to an increase in the number of damaged and dead spermatozoa due to energy deficiency (Solihati *et al.*, 2008 in Nahak *et al.*, 2022). During the dilution and storage process, boar semen is very sensitive to

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temperature changes because the lipid layer on the boar spermatozoa membrane is very thin, making the spermatozoa unable to withstand low temperatures. Spermatozoa metabolism during storage will produce a reaction between the spermatozoa and oxygen, which will cause the formation of free radicals. The free radicals formed will trigger membrane lipid peroxidation, which will reduce the viability and motility of spermatozoa (Sikka, 1996 in Nahak *et al.*, 2022).

The results of the viability test identified live spermatozoa as those that did not absorb color (transparent) in the head, while dead spermatozoa were characterized by color absorption in the head (Figure 1). Spermatozoa viability in these results was still better than the results of a study (Zhang *et al.*, 2009) using a modified Beltsville Thawing Solution and a supplement with 6% (w/v) soy lecithin, which was 59.27±5.80% at - 48 hours of storage, and the results of Sumardani's (2007) study, which was 36.33±1.89% at the 42-hour observation using a modified Zorlesco diluent with fructose at a storage temperature of 18°C.

Spermatozoa Abnormalities

Semen that is still suitable for insemination is semen that has a spermatozoa abnormality rate of <20% (Foeh, 2015). The results of the analysis of variance showed that the effect of prolonged storage in Beltsville Thawing Solution with the addition of 1.0 mM melatonin had no significant effect (P>0.05) on the sperm abnormalities of Berkshire pigs. This was because the antioxidant content of melatonin was able to maintain sperm abnormalities and reduce the increase in abnormalities caused by lipid peroxidation. Based on Duncan's post-hoc test, the highest average abnormality value was found in treatment P3 (6.33±0.70%), followed by P2 (6.22±0.83%) and P1 (6.00±0.86%). The results showed that the percentage of sperm abnormalities reached the highest level of 6.33% in the P3 group stored for 72 hours. This result is still good because, according to Foeh (2015), the percentage of pig sperm abnormalities should only reach 11.1%.

This is because the Beltsville Thawing Solution with added melatonin antioxidant is a diluent that can provide energy for spermatozoa, maintain spermatozoa abnormalities, and reduce the increase in abnormalities caused by simultaneous lipid peroxidation. In general, sperm abnormalities can be caused by various factors, including livestock genetics, stress, environmental temperature, disease, and even handling during semen collection (Arifiantini and Ferdian, 2006). The results of the abnormality examination are presented in (Figure 2.).

Integrity of Intact Plasma Membrane (IPM) of Spermatozoa

The plasma membrane plays an important role in spermatozoa, serving as the primary defense against damage to the external environment in the cell. The function of the plasma membrane is not only limited to protecting the organelles contained in the cell, the plasma membrane can also serve as a potential filter for intra and extracellular substance exchange (Bahmid *et al.*, 2023). The phospholipid structure of the plasma membrane of spermatozoa, which contains highly unsaturated fatty acids, causes spermatozoan cells to be susceptible to free radicals and triggers autocatalytic reactions, which lead to damage to their double bonds (Tamoes *et al.*, 2014).

In the study conducted (Oktaviandari *et al.*, 2025), the average percentage of Intact Plasma Membrane (IPM) of spermatozoa was obtained in the control group whose treatment in the form of Berkshire pig semen was only diluted using BTS® diluent without being given the treatment of adding melatonin compounds, which was 50.33±0.81% which was stored for 48 hours. Meanwhile, based on *Duncan*'s post-hoc test in this study, it was shown that the effect of long-term treatment in Beltsville Thawing Solution diluent with the addition of 1.0 mM of

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melatonin to the intact plasma membrane of Berkshire pig spermatozoa, the highest average Intact Plasma Membrane value was found in the P1 treatment (62.22±0.66%) followed by P2 (57.00±0.86%) and P3 (51.66±1.00%). There was an insignificant difference between treatments ($p>0.05$). These results show that the administration of melatonin to BTS® diluent is able to act as an antioxidant so that it is able to maintain the integrity of the plasma membrane against damage caused by free radicals.

The decrease in the integrity of the plasma membrane of pig spermatozoa is caused by sensitive or unstable pig semen during the storage process due to autocatalytic reactions (chain reactions) which can ultimately damage all phospholipids in the plasma membrane of spermatozoa if not prevented (Prihantoko *et al.*, 2020). Sperm cells react when placed in a hypoosmotic solution, as the hypoosmotic solution enters the cell through the plasma membrane. Due to the difference in osmotic pressure between the solution and the higher osmotic pressure outside the cell, the solution enters the cell and causes swelling. This phenomenon is more easily observed in the tail of the spermatozoa, so it can be counted to determine the integrity of the plasma membrane (Perez-Liano *et al.*, 2001; Zubair *et al.*, 2013). On the other hand, spermatozoa membranes that have been damaged do not experience swelling in the tail (Figure 3.).

Malondyaldehyde (MDA) Spermatozoa

Free radicals can cause oxidative stress, due to the imbalance between oxidants and antioxidants that has the potential to cause cell damage. The reaction of lipid peroxide with free radicals can lead to a decrease in spermatozoa. Lipid oxidation (lipid peroxidase) on the spermatozoan membrane produces MDA compounds, which are toxic to cells so that they cause damage to the spermatozoan membrane and cause a decrease in the integrity of the spermatozoan membrane, thus causing a decrease in sperm quality.

The results of this study showed that the MDA levels of Berkshire pig spermatozoa in BTS® thinner with the addition of the antioxidant melatonin 1.0 mM increased with the increase in storage time at 15–20°C. The highest MDA levels were found consecutively in the P3, P0, and P1 treatments, with values of 2050.53±147.34 ng/ml, respectively; 1979.39±108.76 ng/ml; and 1997.13±134.03 ng/ml. This increase in MDA levels indicates increased lipid peroxidation of the spermatozoa membrane during storage. This phenomenon is in line with the biological characteristics of pig spermatozoa which are highly susceptible to oxidative stress due to the high content of unsaturated phospholipids in the plasma membrane, as explained in the study (Sumardani et al,. 2008) that the phospholipid structure of the porcine spermatozoa membrane is unstable to changes in the storage environment.

Multiple fingerprint analysis showed that the length of *storage of* Berkshire pig semen in BTS® diluent with the addition of 1.0 mM of melatonin had a significant effect ($P<0.05$) on spermatozoa MDA levels. This gradual pattern of improvement suggests that the protective effectiveness of melatonin antioxidants is limited to longer storage durations. These findings can be compared conceptually with studies (Zaetun *et al.*, 2018). During the process of spermatozoa storage, metabolic activity remains active, which physiologically produces reactive oxygen compounds. Physiologically, oxygen plays an important role in metabolic processes by generating energy in mitochondria through redox reactions, which oxidize molecules to produce Adenosine Triphosphate (ATP) as an energy source. During this process, oxygen has several free radical derivatives or ROS (Juan *et al.*, 2021; Martemucci *et al.*, 2022). Free radicals are particularly harmful to the survival of spermatozoa because they have the highly reactive nature of obtaining electrons.

Long-term exposure to oxidative stress will exceed the protective capacity of antioxidants. Therefore, the results of this study confirm that the addition of 1.0 mM melatonin is able to

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protect Berkshire pig spermatozoa for up to 72 hours according to SNI, but does not completely prevent an increase in MDA levels of Berkshire pig spermatozoa during long-term storage, so the storage length needs to be the main consideration in the application of artificial insemination based on pig liquid semen.

CONCLUSIONS AND SUGGESTIONS

Conclusion

BTS® diluent with the addition of 1.0 mM melatonin antioxidant was able to maintain spermatozoa quality for up to 72 hours with a spermatozoa motility percentage value of 40.66%, viability of 53.00%, abnormality of 6.33%, plasma membrane integrity of 51.66%, this result is at the minimum value of the Indonesian National Standard (SNI 8030:2014) which states that the percentage of semen motility suitable for use in artificial insemination (IB) is at least 40%. This shows that melatonin is able to act as an antioxidant that can neutralize free radicals so that it can inhibit the occurrence of oxidative stress and is able to prevent an increase in abnormalities and *malondialdehyde* (MDA) levels of Berkshire pig semen as an indicator of oxidative stress.

Suggestion

Further research needs to be conducted to find alternatives to melatonin content in other ingredients so that they are easy to find in the field. It is recommended for further research to conduct a fertility test using Berkshire pig semen, to find out whether the results of motility, viability, abnormalities, intact plasma membrane and MDA level test obtained are able to cause pregnancy and increase in litter size in pig mothers that will be artificially inseminated using semen from Berkshire pig males.

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Table

Table 1. Mean ± SD results of the effect of storage time on the quality of Berkshire boar sperm diluted using Beltsville Thawing Solution (BTS®) with the addition of 1.0 mM melatonin.

Parameter \ Results	P1	P2	P3
Motility (%)	49.33 ± 0.86[a]	45.44±1.33[b]	40.66±1.22[c]
Viability (%)	61.00 ± 1.11[a]	55.55±1.87[b]	53.00±1.32[c]
Abnormalitas (%)	6.00 ± 0.86[a]	6.22±0.83[a]	6.33±0.70[a]
Plasma Membrane Integrity (%)	62.22 ± 0.66[a]	57.00±0.86[b]	51.66±1.00[c]
MDA Level (ng/ml)	1768.14 ± 95.23[a]	1928.56± 81.58[b]	2070.52±80.54[c]

[a,b,c]Different superscript letters towards the line showed significantly different results (P<0.05). P1 (48-hour observation); P2 (60-hour observation); P3 (72-hour observation). MDA: *Malondialdehyde.*

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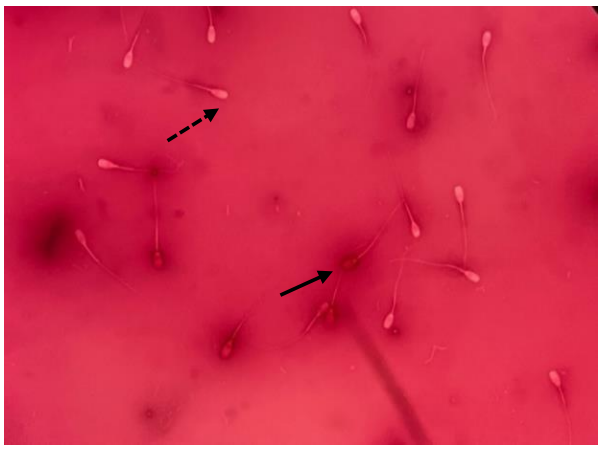

Figure 1. Results of Berkshire pig spermatozoa viability observations. Dead spermatozoa are characterized by spermatozoa that are able to absorb color (arrow), while live spermatozoa are characterized by transparent color or do not absorb color (dashed arrow).

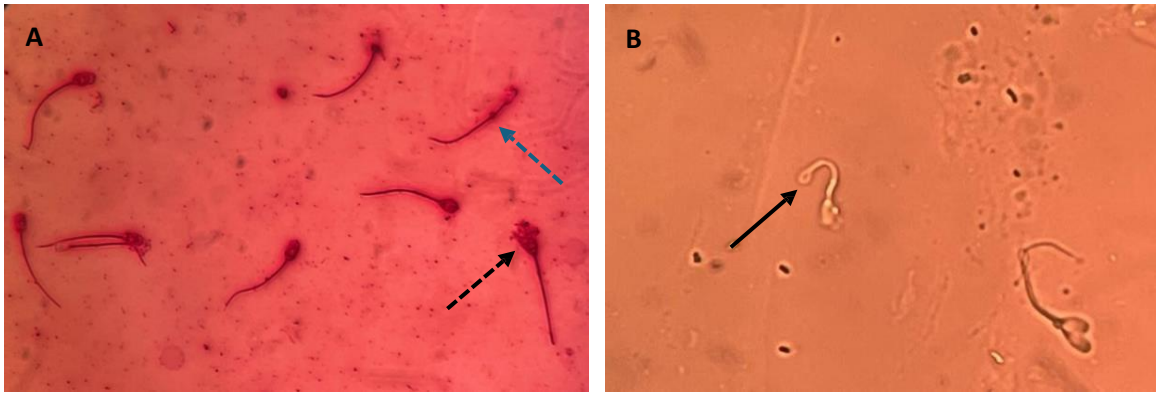

Figure 2. Results of observation of Berkshire pig spermatozoa abnormalities. (A) Spermatozoa exhibiting abnormalities in the form of plasma membrane rupture due to free radicals (dashed arrow) and Spermatozoa exhibiting primary abnormalities characterized by the presence of cytoplasmic droplets (blue dashed arrow). (B) Spermatozoa exhibiting abnormalities in the form of a curled tail (arrow).

Figure 3. Results of observations on the integrity of the plasma membrane of Berkshire pig spermatozoa. Dead spermatozoa are characterized by straight tails (arrows), while live spermatozoa are characterized by folded tails (dashed arrows).

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