Research Article

The Effect of Storage Duration and Melatonin Concentration on BTS Extender on Oxidative Stress and Quality of Landrace Boar Semen

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Abstract | This study aimed to determine malondialdehyde (MDA) levels and the quality of pig semen stored in Beltsville Thawing Solution (BTS) diluent with the addition of various melatonin concentrations. A completely randomized design was adopted, comprising four melatonin concentrations, namely T0 (0 mM (millimolar) as control), T1 (0.5 mM), T2 (1 mM), and T3 (1.5 mM), with each treatment replicated six times. All treatments were stored in a cold box at 15˚C-18˚C and observations were conducted on MDA, Intact Plasma Membrane (IPM), Intact Acrosome Membrane (IAM), Progressive Motility (PM), Viability (V), and Spermatozoa Abnormality (SA) levels at 0, 24, 48, and 72 hours of storage. Data were analyzed using Analysis of Variance and the Duncan test to identify significant differences. The results showed that the diluent treatment group and semen storage time significantly affected ($p < 0.05$) the levels of MDA, IPM, IAM, PM, V, and SA. The BTS diluent supplemented with a 1.0 mM melatonin formula yielded the best sperm quality, with MDA, IPM, IAM, PM, V, and SA levels of 1,919.03ng/ml, 69.42%, 69.67%, 56.08%, 69.17%, and 5.54%, respectively. Additionally, semen quality remained acceptable after 48 hours of storage, with corresponding values of 2,052.49 ng/mL, 54.33%, 54.42%, 43.83%, 52.75%, and 6.88% for the respective parameters. It can be concluded that the addition of optimal melatonin concentration can maintain the quality of boar semen during 48 hours of storage for Artificial Insemination (AI) applications.

Keywords | Pig semen, Storage period, BTS, Melatonin, Sperm quality, IPM, IAM, Progressive motility, Viability, Spermatozoa abnormality

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INTRODUCTION

Bali, Indonesia, has excellent potential for developing pig farming. This is primarily supported by growing customs and culture, culinary tourism, and the region's already famous reputation abroad. This allows farmers to build their business by improving livestock management to improve production and reproduction performance. Efforts to accelerate the improvement of genetic quality and increase the population require implementing Artificial Insemination (AI) technology. A crucial component in the application of AI is the ability to maintain good semen quality and ensure consistent availability. This is essential to maximize the efficiency of semen use and achieve high fertility rates with large litter sizes. The diluent often adopted is Beltsville Thawing Solution (BTS) with the composition of Sodium citrate (0.600g), Glucose (3.700g), Potassium chloride (0.075g), Sodium bicarbonate (0.125g), EDTA

(Ethylene Diamine Tetra Acetate) (0.125g), Streptomycin (100 mg), Penicillin (100,000 IU), and aquadestilate until 100mL (Sokunbi *et al*., 2020). Based on the composition, it primarily contains food sources, buffers, and antibiotics.

Pig semen has a larger volume than that of other domestic animals but is highly susceptible to cold shock and rapid degradation by Reactive Oxygen Species (ROS), leading to reduced fertility. The sensitivity is closely related to the lipid composition, which contains high levels of Polyunsaturated Fatty Acids (PUFAs) such as docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) as well as low cholesterol (Tran *et al*., 2016). During storage, PUFA levels decrease drastically due to the lipid peroxidation process or exposure to ROS. Based on observation, degradation by ROS causes plasma membrane lipid peroxidation, which can produce Malondialdehyde (MDA), a reliable biomarker of oxidative damage (Gusnirwan and Sangging, 2024). MDA causes damage to the spermatozoa membrane and decreases its integrity, thereby reducing sperm quality (Collodel *et al*., 2015; Dutta *et al*., 2019). Degradation by ROS impairs both acrosome and plasma membranes, reduces motility, increases cell permeability, inactivates enzymes, causes cell abnormalities, damages the mid-piece, interferes with the fusion of the egg cell, and leads to cell death (Kurkowska *et al*., 2020). To overcome these problems, semen must be diluted with effective antioxidants to prevent peroxidation during storage.

Melatonin (N-acetyl-5-methoxytryptamine) is a tryptophan derivative, secreted by the pineal gland in the brain. It has an essential function in the neuroendocrine system and plays a vital role in the reproductive system by regulating spermatozoa quality (Lee and Lee, 2023). According to Gonzalez-Arto *et al*. (2016), the synthesis of Melatonin enzymes also occurs in sheep testes. Melatonin also stimulates the activity of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Monteiro *et al*., 2024). In sheep, it has been shown to protect sperm against the harmful effects of ROS and increase motility and membrane integrity during liquid storage of ram semen (Carriço *et al*., 2023; Kumar *et al*., 2022). Melatonin has shown the ability to minimize oxidative damage to spermatozoa caused by ROS and hydroxyl radicals (-OH) as well as counteract nitric oxide radicals (NO) (Makris *et al*., 2023). This natural compound can also eliminate and neutralize free radicals such as hydroxyl anions, peroxyl, and peroxynitrite, reducing sperm mitochondrial oxidative stress (Lee and Lee, 2023). Melatonin can neutralize free radicals and remove them from cells by metabolism into inactive species or inhibit enzyme formation (Monteiro *et al*., 2024). Additionally, it is multifunctional, universal, and soluble in water and lipids, thereby acting as a hydrophilic and hydrophobic antioxidant (Kopustinskiene and Bernatoniene, 2021).

Based on the description above, adding melatonin to the BTS diluent is expected to maintain the pig semen quality. This provides a solution for inseminators to obtain quality semen diluent with adequate storage capacity.

MATERIALS AND METHODS

Ethical Approval, Location and Time of Study

The experimental procedure was reviewed by the Experimental Animal Ethics Committee, Faculty of Veterinary Medicine, Udayana University, and approval was granted under No. 241/UN14.2.9/PT.01.04/2024. The study was conducted between April and May 2024 at the Veterinary Reproduction and Health Laboratory, Faculty of Veterinary Medicine, Udayana University, Denpasar, Bali, Indonesia. The landrace boar was maintained in Baturiti Village, Baturiti Sub-district, Tabanan district, Bali. Analysis of MDA levels was performed at the Joint Laboratory, Faculty of Medicine, Udayana University.

Materials

The materials used included Landrace pig semen and the Porcine Malondialdehyde ELISA kit No. E0151Po (BT LAB, China). Other reagents and chemicals comprised 5g melatonin crystalline (Sigma-Aldrich), D Glucose Anhydrous 250g (Merck, M5250-5GD2-231), Ethylenedinitrilotetraacetic acid/EDTA (Merck, cataloq 108452), Natrium Carbonate 5000g (Merck, 106392 1.06392), Potassium Chloride 1 kg (Merck, 1.04936.1000), Sodium citrate (Merck 1.06448.0500), Sodium bicarbonate 1 kg (Merck, 1.06329.1000), Crystalline NaCl (Merck 1.06404.0500), Fructose (Merck, 1.05323), Eosin Y (Merck, 1.15935.0025), Negrosin (Merck, 1.15924.0025), 70% alcohol, 37% formalin (Merck), Aquabides, Crystalline streptomycin (Meiji, Indonesia) and penicillin (Meiji, Indonesia), as well as sterile gauze.

Instruments

The instruments used included a binocular microscope (CX23 Olympus, Japan), Counting Chamber Neubauer (Improved Assistant Germany), Micropipette (Eppendorf Research Micropipette 3124000121), Pasteur pipette, 10 ml volume pipette, glass object, glass cover, pH paper (Merck pH paper), test tube, measuring cup, Erlen Meyer, test tube rack, water bath (MEMMERT Waterbath 10 L, Sumber Aneka Karya Abadi, Indonesia), one cc and 10 cc syringes, pig semen container, Magnetic Stirrer (OAN LAB Magnetic Stirrer HS-12), and ACIS Digital Scales (BC-500, capacity 500 g, accuracy 0.01 g).

Research Design

A completely randomized design was adopted, with four melatonin concentrations in BTS diluent, namely T0 as control (0 mM), T1 (0.5 mM), T2 (1 mM), and T3 (1.5

mM). Each treatment was repeated six times, and samples were stored in a cold box containing ice packs at 15°C-18°C. Observations were conducted on MDA, IPM, IAM, PM, V, and SA levels at 0, 24, 48, and 72 hours of storage (Bebas *et al*., 2023a).

Pig Semen Storage

In this study, the Landrace pig was the only superior individual in good health, with an age range of 2.5 years. Semen was collected by massage with gloved hands, assisted by a dummy sow (Gadea *et al*., 2020). The surface of the collection bottle was covered with sterile gauze to filter the gel part. Only fraction 2 of the ejaculation was collected, while fractions 1 and 3 were excluded (Stravogianni *et al*., 2022).

Semen Quality Examination

The collected semen was examined macroscopically and microscopically. Macroscopic examination included volume, color, viscosity, odor, and pH, while microscopic examination comprised mass movement, spermatozoa concentration, individual movement, V, and SA (Bebas and Agustina, 2022).

Semen volume was determined by directly reading the scale on the collection tube (Erlenmeyer), and pH was assessed using pH paper. Viscosity was evaluated by tilting the tube and observing the semen's flow along the tube wall. Poor quality was reflected through rapid flow, similar to water. An odor examination was conducted by smelling, and color was observed visually (Bebas *et al*., 2021).

Mass movement was examined by dripping 0.05 ml of fresh semen on a warm glass object (37°C), covered with a glass cover, and observed using a light microscope at 100x magnification. It was scored using the values (++++)= very good, (+++)= good, (++)= moderate, (+)= poor, and (-)= aspermia or no sperm (Bebas *et al*., 2023b). Motility examination was conducted by dripping 0.05 ml of fresh semen on a warm glass object (37 °C), then covered with a glass cover and observed in five fields of view at 400x magnification. The viability and morphology of spermatozoa (normal and abnormal) were evaluated using Eosin-Nigrosin staining with a magnification of 400x (Bebas *et al*., 2023b; Kondracki *et al*., 2017). Live sperm remained transparent, while dead sperm appeared red. Spermatozoa with abnormalities in the head, neck, and tail, or with cytoplasmic droplets, were classified as abnormal. The concentration of spermatozoa was calculated using a Neubauer Hemocytometer (Adu *et al*., 2023). The IPM was observed using the Hypoosmotic Swelling Test (HOST) method. Furthermore, the composition of the HOST solution consisted of 0.49 g of sodium citrate + 0.9 g of fructose, dissolved in aquabides to a volume of 100 ml, producing an osmotic pressure of 100 mOsm/Kg (Kumar *et al*., 2022).

Approximately 20 ml HOST solution was added to 0.2 ml of semen and homogenized, then incubated at 37 °C for 45 minutes. A smear was prepared and evaluated with a magnification of 400x, and at least 200 cells were counted. A circular or bulging tail characterized Spermatozoa with good IPM, while a straight tail characterized those with damaged IPM. IAM examination used 0.9% NaCl solution prepared by dissolving 100 ml of aquabidest. In the process, 1 ml of formalin was added to 99 ml of the solution and homogenized (Adu *et al*., 2023; Bebas *et al*., 2023b). Three parts of a mixture were combined with one part of semen and left for approximately three minutes. Furthermore, 0.05 ml was dropped onto a glass object, covered using a glass cover, and examined under 400x magnification. A total of 100 spermatozoa were evaluated, and those with intact acrosome membranes were identified by the presence of a black acrosome cap (Zhang *et al*., 2021).

Malondialdehyde Examination

The Porcine Malondialdehyde ELISA kit No. E0151Po (BT LAB, China) was adopted to measure MDA levels. This kit was designed to detect MDA in various tissues, serum, plasma, and other biological fluids in pigs. The working principle was based on the binding interaction between the antigen in the tested sample and the antibody coated in the wells. All procedures followed the instructions for the Porcine Malondialdehyde ELISA kit No. E0151Po (BT LAB, China).

Making the BTS Diluent and Adding Melatonin

The BTS diluent formula was prepared with the composition: Sodium citrate (0.600g), Glucose (3.700g), Potassium chloride (0.075g), Sodium bicarbonate (0.125g), EDTA (Ethylene Diamine Tetra Acetate) (0.125g), Streptomycin (100 mg), Penicillin (100,000.IU) and aquadestilate until 100mL, and then homogenized using a magnetic stirrer (Sokunbi *et al*., 2020). Melatonin was added according to the experimental design, with four concentrations: 0, 0.5, 1, and 1.5 mM.

Semen Dilution

BTS diluent and collected semen were placed in a water bath at 37 °C to ensure temperature equilibrium. The semen was then diluted with a diluent made using the following formula:

$$Total\ Volume = \frac{Semen\ volume \times Concentration \times Dose\ volume}{Dose\ concentration}$$

Amount of diluent=Total Volume-Total Semen Volume

The diluted semen was stored in a cold box at 15-18 °C. Observations were conducted on MDA, PM, IPM, MAP, V, and SA levels at 0, 24, 48, and 72 hours of storage.

Data Analysis

The research data were analyzed using Analysis of Variance (ANOVA) with IBM SPSS Statistics 23 for Windows. Variation between treatment groups with $p<0.05$ is considered statistically significant, followed by Duncan's Test.

RESULTS AND DISCUSSION

Results

Macroscopic and microscopic characteristics of fresh Landrace pig semen are presented in Table 1. Meanwhile, the semen quality after treatment with melatonin concentration in the diluent and storage period is shown in Tables 2 and 3. Microscopic images of the plasma membrane, acrosome membrane, abnormality, and dead spermatozoa are detailed in Figure 1.

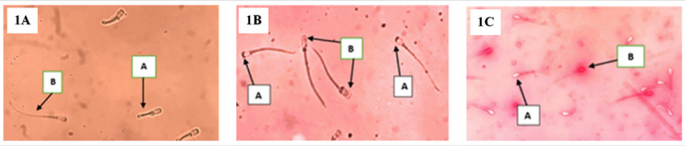

Figure 1: Microscopic evaluation of sperm quality; **A:** Hypo-osmotic swelling test (HOST) for intact plasmamembrane (IPM), where circular tails indicate intact membranes (A) and straight tails indicate damage (B); **B:** Intact acrosome membrane (IAM) assessment, showing intact (A) and damaged (B) acrosomes; **C:** Viability staining with Eosin-Negrosin, where live spermremain unstained (A) and dead sperm absorb red dye (B).

Table 1: Fresh semen quality of landrace pig.

Fresh Semen Quality		
Macroscopic Examination	Semen Viscosity	Moderate
	Semen Color	Milk White
	Semen Volume (ml)	165 ml
	Acidity /pH	7.0
	Odor	Typical Pig
Microscopic Examination	Mass movement	+++
	Concentration (10^6/ml)	825
	Progressive movement (%)	78 P
	Live spermatozoa (%)	90
	Spermatozoa abnormality (%)	4

Description: +++ = The mass wave movement is strong, P = Progressive motility is a sperm's movement forward, and their movements are fast.

Discussion

Table 1 shows that fresh semen macroscopically and microscopically with good quality has a volume, concentration, and progressive motility (PM) of 165 ml, 825 x 10^6 cells/ml, and 78%, respectively. These results are almost the same as Bebas and Gorda (2019), who found that the Landrace pig fresh semen quality, only collected in the second practice, produced a volume, concentration, and PM of 175 ml, 795 × 10^6 cells/ml, and 88%, respectively. This contrasts with Nahak *et al.* (2022), where the semen quality showed values of 230 ml, 230 × 10^6 cells/mL, and 70%, as they obtained a higher volume with a lower concentration and lower progressive motile sperm. The ejaculation results in pigs consists of three fractions, where fractions 1 and 3 are only clear liquids containing a lot of gel, leading to a higher volume with a lower sperm concentration. This shows that the semen used as the sample is of good quality and suitable for proceeding to the next dilution stage. Additionally, suitability criteria included motility ≥70%, concentration ≥200x10^6, and abnormality < 20% (Banamtuan *et al.*, 2021).

Table 2: Melatonin effects on Landrace pig semen quality.

Diluent Treatment	Pig Semen Quality					
	MDA (ng/ml)	IPM (%)	IAM (%)	PM (%)	V (%)	SA (%)
P0 (BTS = Control)	2134.45^a	60.38^a	60.17^a	49.33^a	59.33^a	6.33^a
P1 (BTS + Melatonin 0.5 mM)	1994.66^b	64.00^b	64.67^b	52.75^b	62.88^b	6.38^a
P2 (BTS + Melatonin 1.0 mM)	1919.03^c	69.42^c	69.67^c	56.08^c	69.17^c	5.54^b
P3 (BTS + Melatonin 1.5 mM)	2119.17^a	59.21^d	58.50^d	49.17^a	59.63^a	6.75^a

Description: Superscripts with different letters in the column represented significant differences ($p<0.05$). MDA (Malonaldehyde), IPM (Intact Plasma Membrane), IAM (Intact Acrosome Membrane), PM (Progressive Motility), V (Viability), and SA (Spermatozoa Abnormality).

Based on Table 2, the diluent treatment group has a significant effect ($p<0.05$) on MDA, IPM, IAM, PM, V, and SA levels. After further analysis using the Duncan Test, treatment P2 produces the lowest significant MDA ($p<0.05$), the highest IPM ($p<0.05$), the highest IAM, the highest PM ($p<0.05$), the highest V ($p<0.05$), and the significant SA ($p<0.05$) respectively at 1,919.03 (ng/ml), 69.42%, 69.67%, 56.08%, 69.17%, and 5.54% compared to P0, P1, and P3.

Adding melatonin with a concentration of 1.0 mM to the BTS diluent gives the most optimal effect on the pig semen quality during storage. A concentration of 0.5 mM has not provided an optimal effect, while adding 1.5 mM reduces the impact due to excessive concentration. According to analysts from Kansas State University, America, high doses of antioxidants can be counterproductive. Antioxidants are effective only in the presence of pro-oxidant compounds that initiate oxidation. When antioxidant levels exceed those of pro-oxidants, the body may respond by generating pro-oxidants to maintain balance, forming irreparable

free radicals (Sotler *et al*., 2019). Karepu *et al*. (2020) stated that adding excessive antioxidant compounds harmed toxicity, thereby becoming pro-oxidants. Based on definitions, pro-oxidants promote oxidation in cell components. This theory aligns with the results that the P3 treatment group, given 1.5 mM melatonin in the BTS diluent, decreased semen quality. Similar research was conducted by Bebas and Gorga (2016), who added the antioxidant astaxanthin to the egg yolk phosphate diluent of various types of poultry with concentrations of 0%, 0.002%, 0.004% and 0.008% to dilute pig semen, it turned out that the concentration of 0.002% was able to maintain the quality of pig semen the best when compared to concentrations of 0.004 and 0.008%.

Table 3: Effect of storage time on the quality of landrace pig semen.

Storage Period Treatment	Pig Semen Quality					
	MDA (ng/ml)	IPM (%)	IAM (%)	PM (%)	V (%)	SA (%)
0 Hours	1995.05^{a}	88.21^{a}	87.79^{a}	77.08^{a}	88.38^{a}	4.83^{a}
24 Hours	2010.53^{a}	65.58^{b}	65.83^{b}	52.13^{b}	64.50^{b}	5.58^{b}
48 Hours	2052.49^{a}	54.33^{c}	54.42^{c}	43.83^{c}	52.75^{c}	6.88^{c}
72 Hours	2109.23^{b}	44.88^{d}	44.96^{d}	34.29^{d}	45.36^{d}	7.71^{d}

Description: Different superscript letters within a column indicate significant differences ($p < 0.05$). MDA (Malondialdehyde), IPM (Intact Plasma Membrane), IAM (Intact Acrosomal Membrane), PM (Progressive Motility), V (Viability), and SA (Spermatozoa Abnormality).

Melatonin is a tryptophan derivative, secreted by the pineal gland in the brain, which has an essential function in the neuroendocrine system. Furthermore, it plays a vital role in the reproductive system in regulating spermatozoa quality. Melatonin can improve semen quality in small ruminants such as sheep (Casao *et al*., 2010; Succu *et al*., 2011; Budiyanto *et al*., 2024). In cattle, it has been shown to protect sperm against the harmful effects of ROS as well as increase motility and membrane integrity during liquid semen storage (Kumar *et al*., 2022). Melatonin minimizes oxidative damage to spermatozoa caused by ROS and hydroxyl radicals (OH), as well as counteracts nitric oxide (NO) radicals (Makris *et al*., 2023). This tryptophan derivative can also eliminate and neutralize free radicals such as hydroxyl anions, peroxyl, and peroxynitrite, as well as reduce sperm mitochondrial oxidative stress caused by ROS (Lee and Lee, 2023). Melatonin can directly neutralize free radicals and remove them from cells by metabolism into inactive species or inhibiting the forming enzymes (Manchester *et al*., 2015). The compound is multifunctional, universal, as well as soluble in water and lipids, thereby acting as a hydrophilic and hydrophobic antioxidant (Hardeland, 2013; Budiyanto *et al*., 2024).

Based on the results, the storage period treatment has a significant effect ($p < 0.05$) on MDA, IPM, IAM, PM, viability, and SA levels. Following Duncan's further test, a significant decrease in semen quality ($p < 0.05$) was observed from 0, 24, 48, and 72 hours of storage, as presented in Table 3. Based on the eligibility of liquid pig semen that can be used for AI after storage, it has PM ≥40% with a minimum spermatozoa concentration of 2,500 million (SNI, 2023). Therefore, in this study, semen is suitable for use only for 48 hours of storage with MDA, IPM, IAM, PM, Viability, and SA levels of 2,052.49 (ng/ml), 54.33%, 54.42%, 43.83%, 52.75%, and 6.88%, respectively.

The decrease in semen quality is in line with the length of the storage process. The viability of fresh semen (90%), decreased to 88.38% during 0 hours of storage, this is because fresh semen before being stored undergoes a macroscopic and microscopic semen assessment process. The semen dilution process with a diluent is then stored at a cold temperature ranging from 15oC-20oC, currently called 0-hour storage. Semen from the time of storage, evaluation, and cold storage has undergone a peroxidation process which causes its viability to decrease. Pig spermatozoa are very sensitive to cold shock and quickly experience ROS attacks, closely related to the composition of plasma membrane lipids containing high levels of PUFA such as DHA and DPA, and low cholesterol (Tran *et al*., 2016). During storage, PUFA decreases drastically due to ROS attack or the lipid peroxidation process. ROS attacks result in plasma membrane lipid peroxidation, which can increase MDA as a marker of cell membrane damage due to the peroxidation process (Ayala et al., 2014). Furthermore, the attack triggers loss of membrane integrity in both IAM and IPM, decreased PM, increased SA, increased cell permeability, enzyme inactivation, damage to the mid-piece, fusion of egg cells, and cell death (Kurkowska *et al*., 2020).

The decrease in pig semen quality during storage is caused by a lack of energy and an increase in the number of damaged and dead spermatozoa (Bebas *et al*., 2023b). Spermatozoa metabolism during storage produces redox reactions in mitochondria that cause the formation of free radicals. These radicals trigger membrane lipid peroxidation, which leads to elevated MDA levels, damage to the plasma and acrosome membrane, decreased PM, and increased abnormality (Agarwal *et al*., 2014).

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, supplementing the BTS diluent with 1.0 mM melatonin significantly improves semen quality by reducing oxidative stress (MDA levels) and maintaining sperm motility, membrane integrity, and viability during

storage. Semen stored under these conditions remains viable for up to 48 hours, meeting the minimum standards for artificial insemination (AI). These findings highlight melatonin's potential as an antioxidant in semen extenders, offering a practical solution for improving reproductive efficiency in pig farming. However, further studies should explore the effects of melatonin in larger bar populations and extended storage durations to validate its broader applicability.

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NOVELTY STATEMENTS

This article presents a novelty in the use of melatonin as a natural endogenous antioxidant in extenders to improve the quality of boar semen during storage. This study shows that melatonin at a concentration of 1.0 mM can reduce oxidative stress, enhance mitochondrial and sperm DNA protection, and maintain semen quality. These findings provide a new concept in the development of extenders and procedures for storing boar semen.

AUTHOR'S CONTRIBUTIONS

Wayan Bebas prepared the study concept and design and supervised the entire implementation. Komang Ngurah Suarbawa was in charge of pig maintenance and semen collection. I. Made Merdana worked on the retailer formulation and MDA examination. Wayan Bebas, Komang Ngurah Suarbawa, and I. Made Merdana together participated in the examination of semen quality, data collection, data analysis, and interpretation, as well as writing the publication manuscript.

Conflict of Interest

The authors declare no conflict of interest in the publication of this study.

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