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Post-Thaw Semen Quality of Duroc Boars Supplemented with Trehalose and Glycerol in Beltsville Thawing Solution and Tris-Yolk Extenders

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Abstract:

This study aimed to evaluate the quality of post-thawed semen in Duroc boars following supplementation with trehalose and glycerol as cryoprotectants in different extenders. The extenders used were Beltsville Thawing Solution (BTS) and Tris-Aminomethane Egg Yolk (Tris-Yolk). Semen was collected from a 2.5-year-old Duroc boar using the massage technique. Fresh semen was evaluated macroscopically and microscopically, and only ejaculates with motility >75% and sperm concentration >200 × 10^6/mL were considered suitable for cryopreservation. Semen was initially diluted using BTS or Tris-Yolk extenders and allocated into four tubes, then maintained at room temperature (20–22°C) for two hours (holding time). Afterward, centrifugation was performed to separate the pellet from the supernatant. The resulting pellet was resuspended with extender supplemented with 100 mM trehalose and 4% glycerol. The semen was equilibrated at 5°C for two hours and packed into 0.5 mL straws before freezing in liquid nitrogen vapor. Post-thaw (PT) evaluation showed that sperm motility in BTS supplemented with trehalose and glycerol was significantly higher (25.88 ± 0.72) compared to BTS without supplementation (21.53 ± 0.76), Tris-Yolk with supplementation (16.56 ± 1.11), and Tris-Yolk without supplementation (15.14 ± 0.88) ($P < 0.05$). These findings suggest that the combination of 100 mM trehalose and 4% glycerol enhances cryopreservation outcomes by better preserving sperm motility post-thaw, particularly in the BTS extender. In conclusion, trehalose and glycerol supplementation in BTS extender provides a more effective cryoprotective environment for maintaining the post-thaw motility of Duroc boar sperm compared to Tris-Yolk extender or non-supplemented extenders.

Keywords — **Artificial insemination, Beltsville Thawing Solution (BTS), cryopreservation, Duroc boar, semen extender**

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I. INTRODUCTION

The pig farming sector in Indonesia continues to grow across various regions, serving not only as a source of meat but also for cultural and traditional purposes. In 2015, the pig population in Indonesia was recorded at 7.81 million heads. This number increased until 2019 but experienced a decline in 2020 due to an outbreak of African Swine Fever (ASF), before rising again to 8.01 million in 2021 [1]. Artificial Insemination (AI) has become a

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widely adopted reproductive biotechnology aimed at improving genetic quality through the deposition of semen from genetically superior boars into the female reproductive tract using specific techniques [2].

Although liquid semen is predominantly used for AI in pigs, frozen semen offers several advantages, including long-term storage, efficient reproduction management, and broader dissemination of superior genetics, even across international borders [3]. Frozen semen also plays a significant role in conserving genetic diversity, particularly among rare pig breeds [4]. However, boar sperm is highly sensitive to cold shock due to the high content of phospholipids and polyunsaturated fatty acids in the plasma membrane, making it susceptible to cryodamage during freezing [5].

To mitigate cold shock and improve post-thaw semen quality, selecting an appropriate extender and optimal cryoprotectant concentration is crucial. Beltsville Thawing Solution (BTS) is a widely used commercial short-term extender capable of maintaining boar semen quality for up to 3 days [6]. Cryoprotectants play a vital role in protecting sperm cells during the cryopreservation process. Glutathione, for instance, has been shown to maintain sperm motility post-thaw [7]. Trehalose acts as an extracellular cryoprotectant, while glycerol functions intracellularly to prevent membrane damage [8].

The use of cryoprotectant-supplemented extenders has proven beneficial in reducing sperm damage during freezing [9]. These compounds, including sugars such as trehalose, also help stabilize osmotic pressure and provide energy for sperm survival during preservation [10]. Thus, optimizing extender formulation and cryoprotectant supplementation is essential to enhance the viability and functionality of frozen-thawed boar semen.

II. MATERIALS AND METHOD

This section describes the materials and methods employed in this study.

A. Research Period and Location

This research was conducted from October to December 2024 at the Regional Artificial Insemination Center (Balai Inseminasi Buatan Daerah) located in Baturiti, Tabanan Regency, Bali Province, Indonesia.

B. Research Materials

This study utilized fresh semen collected from a single 2.5-year-old Duroc boar (ID: CAPOH 02) weight : 240 kg. The boar was housed in an individual pen equipped with a feeder and water dispenser. The boar was fed 4 kg of concentrate per head per day, with water provided ad libitum. Semen collection was performed twice a week using a dummy sow, resulting in a total of 10 ejaculates. Only ejaculates exhibiting sperm motility greater than 80% were selected for cryopreservation.

C. Research Method

This study employed a laboratory-based experimental method using a Completely Randomized Design (CRD) with four treatments and ten replications, resulting in a total of 40 experimental units. The treatments were as follows: T0: BTS (control) ; T1: BTS + 100 mM Trehalose ; T2: Tris-Aminomethane Egg Yolk (without Trehalose) ; T3: Tris-Aminomethane Egg Yolk + 100 mM Trehalose.

D. Preparation of Egg Yolk

The egg yolk used in this study was obtained from fresh, high-quality commercial chicken eggs. The eggshells were cleaned using alcohol before cracking to separate the yolk from the albumen. The yolk was gently poured onto filter paper and tilted to allow the remaining egg white to be absorbed. The purified yolk was then transferred into a graduated cylinder. It was mixed with distilled water at a ratio of 1:2 (15 mL yolk : 30 mL distilled water) and homogenized for 15 minutes. The homogenized yolk solution was centrifuged at 2,000 rpm for 15 minutes. The supernatant was collected, and the pellet was discarded. The resulting clarified egg yolk was then ready for use.

E. Preparation of Basic Extender Using Beltsville Thawing Solution (BTS)

Beltsville Thawing Solution (BTS; Minitub, Germany) is a standard extender commonly used for boar semen dilution. The extender was prepared by dissolving 2.5 grams of BTS powder in 50

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milliliters of distilled water (Water for Injection) and homogenized thoroughly. Once homogenized, egg yolk was added to the BTS solution at a ratio of 1:4 (10 mL egg yolk : 40 mL BTS extender). The BTS–egg yolk mixture was then homogenized for 15 minutes. The final extender solution was warmed in a water bath at 37°C before use.

F. Preparation of Tris-Aminomethane-Based Extender

The Tris-Aminomethane-based extender was prepared using the following components: 1.82% Tris (hydroxymethyl aminomethane), 0.98% citric acid, and 0.25% fructose, all dissolved in 50 mL of distilled water. Then, 10 mL of egg yolk was mixed with 40 mL of the prepared Tris extender and homogenized for 15 minutes. The resulting Tris–egg yolk extender was warmed in a water bath at 37°C. Once warmed, 0.8 mL of penicillin and 0.7 mL of streptomycin were added as antibiotics and the solution was homogenized again for 10 minutes. The Tris–egg yolk extender was then ready for use.

G. Freezing Procedure Using BTS–Egg Yolk Extender

BTS–egg yolk extender was mixed with fresh semen that met the quality requirement of >75% motility, at a dilution ratio of 1:1 (semen:extender). The diluted semen was then subjected to a holding time for 2 hours at room temperature (20–22°C). After the holding period, the semen was centrifuged at 2,000 rpm for 15 minutes to separate the pellet from the supernatant.

H. Freezing Procedure Using Tris–Egg Yolk Extender

In the first dilution step, fresh semen was diluted with Tris–egg yolk extender at a 1:1 ratio. The diluted semen was then subjected to a holding time to equilibrate the temperature between the semen and the extender. This holding time was maintained for 2 hours at room temperature (20–22°C). After holding, the semen was centrifuged at 2,000 rpm for 15 minutes to separate the pellet from the supernatant.

In the second dilution step, the resulting pellet was resuspended in extender supplemented with cryoprotectants: 100 mM trehalose and 4% glycerol. The cryoprotectant-supplemented semen was stored in centrifuge tubes and equilibrated at 5°C for 2 hours. Following equilibration, the semen was packed into 0.5 mL straws at a concentration of 200 million spermatozoa per 0.5 mL.

The straws then placed on a freezing rack positioned 5 cm above the surface of liquid nitrogen within a Styrofoam box for 20 minutes. The final freezing step was completed by plunging the straws into a liquid nitrogen container for long-term storage and subsequent evaluation.

I. Parameters

The parameters observed in this study included post-thaw motility, viability, abnormalities, and plasma membrane integrity. These were analyzed to evaluate the effects of cryoprotectants on frozen boar semen.

Motility: Post-thaw motility was assessed by thawing semen straws in a water bath at 37°C for 30 seconds. The motility evaluation was conducted using a light microscope by observing and counting the number of progressively motile spermatozoa versus non-progressive ones.

Viability: Sperm viability was determined using eosin-nigrosin staining to distinguish between live and dead spermatozoa. Live sperm cells did not absorb the stain and appeared translucent, whereas dead sperm absorbed the stain and appeared colored.

Abnormalities: Sperm abnormalities were identified as structural defects, such as detached heads, double heads, broken tails, coiled tails, and bifurcated tails. Abnormalities were examined microscopically using eosin-nigrosin-stained slides.

Plasma Membrane Integrity: Plasma membrane integrity (PMI) refers to the condition in the sperm plasma membrane remains undamaged after cryopreservation. The Hypo-Osmotic Swelling Test (HOST) was used to assess PMI, using a solution containing 0.9 g fructose and 0.49 g sodium citrate dissolved in 100 mL distilled water. A volume of 0.01 mL semen was mixed with 1 mL of HOST solution and homogenized. The integrity of the plasma membrane was evaluated microscopically using a smear technique from the mixture.

J. Data Analysis

The data were analyzed using Analysis of Variance (ANOVA) under a Completely Randomized Design (CRD). When significant differences were detected ($P < 0.05$), the analysis

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was followed by a Chi-square test based on the Indonesian National Standard (SNI). All statistical analyses were performed using R Studio software.

III. RESULTS AND DISCUSSION

Fresh Semen Quality

The evaluation of fresh semen quality was conducted to determine its suitability for further processing and cryopreservation. The characteristics of fresh semen are presented in Table 1, with the mean semen volume recorded at 142.0 ± 15.5 mL. This average differs from the findings of [11], who reported volumes ranging from 240 to 320 mL, but is relatively similar to the results of [12], who reported a mean volume of 167.5 ± 19.84 mL. Several factors may influence variations in macroscopic semen characteristics, including boar age, ejaculation frequency, and the level of sexual stimulation during collection.

TABLE I
FRESH SEMEN QUALITY OF DUROC BOAR

Semen Quality Parameters	Mean ± SD
Color	Milky-white
Ph	7.8 ± 0
Odor	Characteristic semen odor
Volume (mL)	142.0 ± 15.5
Motility (%)	79.5 ± 1.58
Viability (%)	81.23 ± 0.59
Concentration (×10⁶ cells/mL)	272.3 ± 15.83
Abnormalities (%)	5.72 ± 0.22

Motility

Table 2 shows a significant difference in post-thaw motility (PTM) percentages among the treatment groups. Treatment T1 exhibited the highest motility (25.88 ± 0.72[a]), indicating the best performance. Although T0 showed lower motility than T1, it performed better than T2 and T3, both of which exhibited a significant decrease in sperm motility. The supplementation of 100 mM trehalose and 4% glycerol as cryoprotectants had a noticeable impact on the motility of frozen-thawed semen. Cryopreservation using BTS–egg yolk extender supplemented with trehalose and glycerol proved more effective than extenders without cryoprotectants.

Trehalose functions as an extracellular cryoprotectant, while glycerol serves as an intracellular cryoprotectant. The combination of 100 mM trehalose and glycerol in BTS extender was reported to result in the highest sperm motility (30.00 ± 4.47%) compared to other extenders (Yusuf et al., 2017). Furthermore, BTS–egg yolk extender supplemented with trehalose and glycerol can offer better protection against cryodamage to spermatozoa than extenders lacking such supplementation [13].

TABLE II
MEAN SPERM MOTILITY OF FROZEN DUROC BOAR SEMEN (%)

Treatment	Fresh	Equilibration (5◦C)	Post- Thaw Motility
T0	80.01 + 0.52[a]	70.27 + 2.55[b]	21.53 + 0.76[b]
T1	79.83 + 0.43[a]	73.07 + 2.20[a]	25.88 + 0.72[a]
T2	80.21 + 0.46[a]	60.02 + 2.97[d]	15.14 + 0.88[d]
T3	79,63+ 0.46[a]	63.23 + 3.25[c]	16.56 + 1.11[c]

Different superscript letters (a–d) in the same column indicate significant differences (P < 0.05). T0: BTS (control) ; T1: BTS + 100 mM Trehalose ; T2: Tris-Aminomethane Egg Yolk (without Trehalose) ; T3: Tris-Aminomethane Egg Yolk + 100 mM Trehalose.

Statistical analysis showed significant differences in sperm motility across all treatment stages (P < 0.05). The highest sperm motility was observed in T1, both in the fresh state (73.07 ± 2.20%), after equilibration at 5°C (36.68 ± 1.26%), and post-thawing (25.88 ± 0.72%). In contrast, T2 showed the lowest motility across all stages—fresh (60.02 ± 2.97%), after equilibration (23.17 ± 1.51%), and post-thaw (15.14 ± 0.88%). A reduction in motility was also observed in T3, which recorded lower values than T0 and T1, but higher than T2. Motility in the control group (T0) was lower than in T1, yet remained superior to both T2 and T3. These findings indicate that P1 treatment is the most effective in preserving sperm motility during the freezing process compared to other treatments.

The motility graph also demonstrates significant differences at various storage temperatures. A decrease in motility was observed at each stage for all treatments, highlighting the critical role of

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storage temperature in maintaining sperm function. As shown in Figure 1, no significant difference was found in fresh semen motility across treatments, with an average of 79.5%, indicating that all ejaculates were suitable for further cryopreservation. Semen was processed using different extenders and maintained at room temperature (20–22°C), allowing gradual adaptation to lower temperatures prior to freezing. The equilibration phase at 5°C is a critical stage where spermatozoa adapt to the extender composition, including cryoprotectants such as trehalose and glycerol [14].

A marked decrease in motility was observed during the post-thaw stage, with reductions ranging from 45–47%. Reported post-thaw motility in boar semen varies considerably, from 46.2 ± 1.3% [15], 26.5 ± 1.9% to 40.7 ± 1.8% [16], and 21.67 ± 7.53% to 30.00 ± 4.47% [17].

The thawing process performed at 37°C for 20 seconds can induce significant damage to the sperm plasma membrane. As the membrane regulates ion transport and motility, its structural integrity is essential for proper sperm function. Thawing-related membrane damage may impair these functions and has been reported to affect 40–50% of spermatozoa [18].

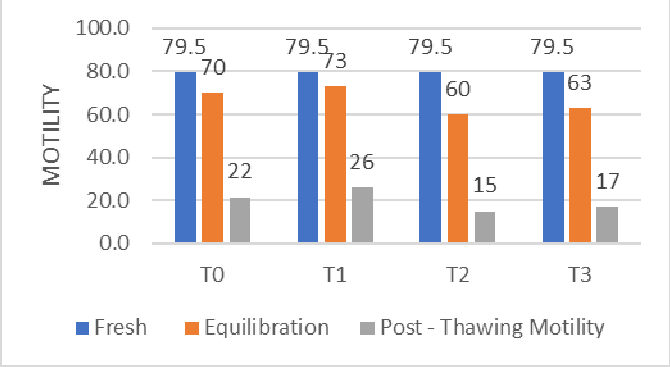

Fig. 1 Mean Sperm Motility of Frozen Duroc Boar Semen (%)

Viability

Statistical analysis presented in Table 3 indicates significant differences in sperm viability across treatment stages ($P < 0.05$). The highest viability in the fresh condition was observed in treatment T1 (77.97 ± 2.94%), while the lowest was recorded in T2 (72.45 ± 2.56%). During the equilibration stage at 5°C, a reduction in viability occurred across all treatments. T1 maintained the highest viability (62.29 ± 2.92%), which was not significantly different from T0 (60.95 ± 1.59%), but was significantly higher than T2 (56.81 ± 2.13%) and T3 (58.22 ± 2.00%).

The decrease in viability continued after the thawing process. T1 remained the most effective treatment, showing the highest post-thaw viability (57.18 ± 2.79%), while T2 demonstrated the lowest value (49.35 ± 2.77%). These results suggest that T1 treatment is the most effective in preserving sperm viability during the cryopreservation process.

The reduction in viability at the post-thaw stage is likely associated with structural damage to the sperm plasma membrane during freezing. Such damage compromises membrane function, particularly its protective role, thereby reducing the overall viability of the spermatozoa [19].

TABLE IIII
MEAN SPERM VIABILITY OF FROZEN DUROC BOAR SEMEN (%)

Treatment	Fresh	Equilibration (5◦C)	Post- Thaw Motility
T0	81.67 ± 1.74^{a}	76.019 ± 2.32^{b}	54.205 ± 1.62^{b}
T1	81.16 ± 2.36^{a}	77.965 ± 2.94^{a}	57.180 ± 2.79^{a}
T2	81.90 ± 1.99^{a}	72.445 ± 2.56^{d}	49.348 ± 2.77^{c}
T3	80.94 ± 1.73^{a}	74.509 ± 2.74^{c}	50.770 ± 1.70^{c}

Different superscript letters (a–d) in the same column indicate significant differences ($P < 0.05$). T0: BTS (control) ; T1: BTS + 100 mM Trehalose; T2: Tris-Aminomethane Egg Yolk (without Trehalose) ; T3: Tris-Aminomethane Egg Yolk + 100 mM Trehalose.

The mean viability graph (Figure 2) of frozen semen demonstrated a decreasing trend across all treatments. The figure showed no significant differences in fresh semen viability among the treatments, with an average viability of approximately 81%. The highest viability was recorded in T1, with mean values of 78% at the equilibration stage and 57% post-thaw. Conversely, the lowest viability was observed in T3, with averages of 72% at equilibration and 49% post-thaw.

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Gradual temperature changes, from equilibration to post-thawing (PTM), likely influenced membrane integrity and the metabolic activity of spermatozoa. The composition of the extender and cryoprotectants in T1 appeared more effective in maintaining spermatozoa condition compared to the other treatments. BTS® extender contains glucose as an energy source; EDTA, which regulates calcium levels in the solution; antibiotics (penicillin and streptomycin) to suppress bacterial growth; buffering agents such as sodium citrate and sodium bicarbonate; and potassium chloride, which serves to depress or anesthetize spermatozoa [20].

Trehalose, a disaccharide sugar, has demonstrated greater cryoprotective efficacy compared to monosaccharide sugars, enhancing its effectiveness in preserving sperm viability during cryopreservation.

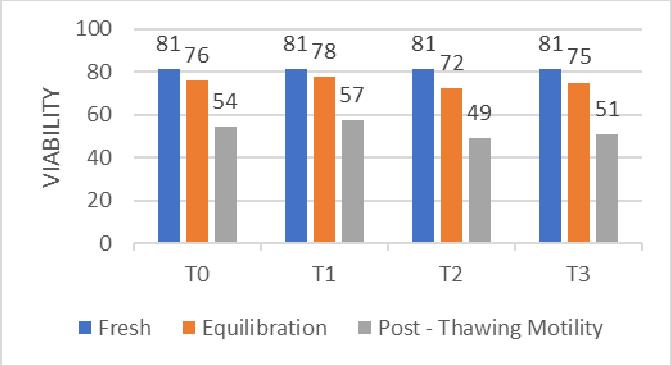

Fig. 2 Mean Sperm Viability Of Frozen Duroc Boar Semen (%)

Abnormality

Sperm abnormalities in frozen semen are a critical parameter in evaluating boar semen quality. Table 4 shows that supplementation with trehalose and glycerol had a significant effect on the percentage of abnormalities across treatments. The highest abnormality rate was observed in treatment T3, with a mean post-thaw abnormality of 9.49 ± 0.88^{a} %.

This condition is likely caused by extreme temperature changes and oxidative stress during the freezing and thawing processes. Oxidative stress, triggered by the accumulation of reactive oxygen species (ROS), can lead to DNA damage and sperm dysfunction [21].

TABLE IV
MEAN SPERM ABNORMALITY OF FROZEN DUROC BOAR SEMEN (%)

Treatment	Fresh	Equilibration (5◦C)	Post- Thaw Motility
T0	5.58 ± 0.54^{a}	7.22 ± 7.22^{c}	8.37 ± 0.84^{c}
T1	5.80 ± 0.43^{a}	7.06 ± 7.06^{d}	7.94 ± 0.75^{d}
T2	5.78 ± 0.50^{a}	7.34 ± 7.34^{b}	9.00 ± 0.86^{b}
T3	5.70 ± 0.69^{a}	7.46 ± 7.46^{a}	9.49 ± 0.88^{a}

Different superscript letters (a–d) in the same column indicate significant differences (P < 0.05). T0: BTS (control) ; T1: BTS + 100 mM Trehalose ; T2: Tris-Aminomethane Egg Yolk (without Trehalose) ; T3: Tris-Aminomethane Egg Yolk + 100 mM Trehalose.

Statistical analysis showed significant differences in sperm abnormalities across all treatments and storage temperatures. In the fresh condition, the highest abnormality was observed in T3 (7.46 ± 0.74), followed by T2 (7.34 ± 0.73), T0 (7.22 ± 0.72), and the lowest in T1 (7.06 ± 0.71). During the equilibration phase at 5°C, the highest abnormality was again recorded in T3 (8.33 ± 0.73), followed by T2 (8.04 ± 0.72), T0 (7.68 ± 0.74), and T1 (7.47 ± 0.67). A similar trend was observed in the post-thaw condition, with T3 showing the highest abnormality (9.49 ± 0.88), followed by T2 (9.00 ± 0.86), T0 (8.37 ± 0.84), and T1 (7.94 ± 0.75) with the lowest abnormality rate.

These results indicate that T1 consistently demonstrated the lowest level of sperm abnormalities across all stages, suggesting its superior ability to preserve sperm structural integrity during cryopreservation.

The Figure 3 showed a clear trend of increased sperm abnormalities as storage temperature decreased. The lowest abnormalities were recorded in fresh semen, while the highest were observed post-thaw (PTM), highlighting that both cold storage (5°C) and freezing-thawing processes contribute to increased morphological damage. The greater the temperature change between storage stages, the higher the abnormality rate.

This trend is largely attributed to cold shock, which induces structural changes in the sperm plasma membrane, particularly in the phospholipid bilayer [22]. Cold shock can disrupt osmotic

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balance and lead to physical damage, such as detached or broken sperm heads and tails [23].

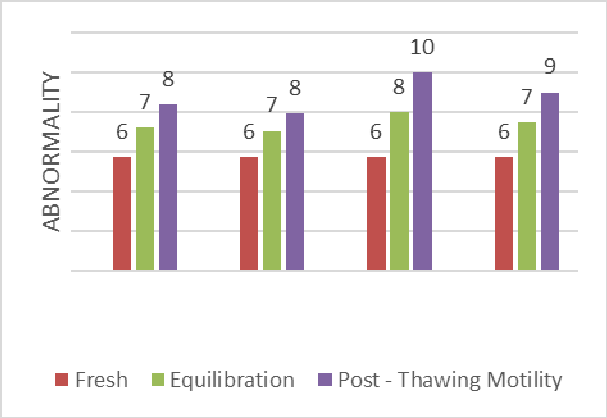

Fig. 3 Mean Sperm Abnormaliry Of Frozen Duroc Boar Semen (%)

Plasma Membrane Integrity

Table 5 demonstrates significant differences in plasma membrane integrity among the treatments. T1 consistently showed the highest mean percentage of intact plasma membranes across all evaluated variables. In contrast, T2 recorded the lowest values, which were even lower than those reported by [24], who found a mean membrane integrity of 56.69 ± 5.64% using Tris–egg yolk extender.

An appropriately formulated extender combined with effective cryoprotectants supports the sperm's ability to maintain plasma membrane integrity during cryopreservation. The combination of trehalose and glycerol as cryoprotectants had a significant effect in preserving membrane integrity, compared to the use of glycerol alone [25]. T2, which utilized Tris–egg yolk extender without trehalose and glycerol, resulted in the lowest membrane integrity among all treatments.

Trehalose supplementation in Tris–egg yolk extenders has been shown to improve plasma membrane integrity compared to extenders without trehalose [26]. Moreover, glycerol inclusion in the extender provides additional protection to spermatozoa by reducing membrane damage during cryopreservation.

TABLE V
MEAN PLASMA MEMBRAN INTEGRITY OF FROZEN DUROC BOAR SEMEN (%)

Treatment	Equilibration (5◦C)	Before Freezing	Post- Thaw Motility
T0	67.43 ± 0.86^{b}	59.35 ± 0.76^{b}	45.11 ± 0.58^{c}
T1	68.86 ± 0.88^{a}	61.28 ± 0.78^{a}	54.54 ± 0.69^{a}
T2	64.92 ± 1.06^{c}	52.90 ± 0.68^{d}	44.97 ± 0.57^{d}
T3	66.79 ± 0.85^{b}	57.44 ± 0.73^{c}	49.40 ± 0.63^{b}

Different superscript letters (a–d) in the same column indicate significant differences ($P < 0.05$). T0: BTS (control) ; T1: BTS + 100 Mm Trehalose; T2: Tris-Aminomethane Egg Yolk (without Trehalose) ; T3: Tris-Aminomethane Egg Yolk + 100 mM Trehalose.

The Figure 4 indicates that plasma membrane integrity (PMI) was highest during the equilibration stage at 5°C and declined significantly post-thaw. Variations in PMI were observed across treatments, with P1 showing the highest values, followed by a gradual decline in P2 and a slight increase in P3. Sperm membrane stability at the equilibration stage remained relatively higher compared to the post-thaw stage, where integrity decreased more substantially.

Fluctuations in plasma membrane integrity among treatments suggest that BTS extender supplemented with trehalose and glycerol effectively preserves PMI. BTS is a widely used extender in boar semen cryopreservation, known for its buffering composition that maintains ionic and pH balance. It contains glucose, citrate, bicarbonate, EDTA, and potassium chloride (KCl), which together form a buffering system essential for maintaining ionic homeostasis in spermatozoa during cryopreservation [27].

In contrast, the Tris–egg yolk extender without trehalose and glycerol yielded the lowest PMI values, likely due to insufficient membrane protection. A slight improvement was noted when trehalose and glycerol were added to the Tris extender, although the results remained inferior to those of BTS. This difference may be attributed to the distinct buffering capacities of BTS and Tris, which influence the sperm's ability to withstand temperature-induced stress. BTS, with a citrate-based buffer system, exhibits different buffering

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characteristics compared to Tris, thus affecting the spermatozoa's response to cryogenic conditions [28].

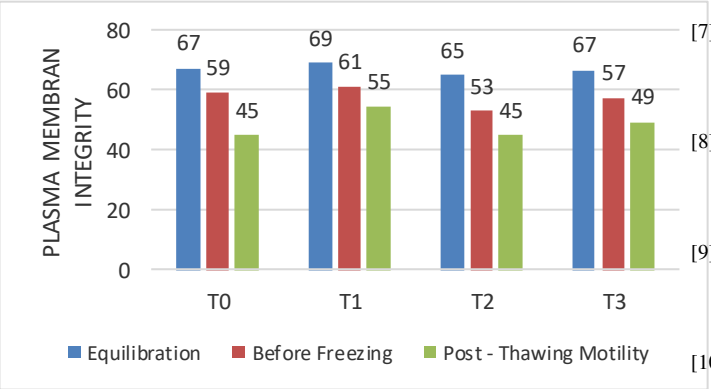

Fig. 4 Mean Plasma Membran Integrity Of Frozen Duroc Boar Semen (%)

IV. CONCLUSIONS

Cryopreservation using BTS–egg yolk extender supplemented with trehalose and glycerol effectively preserved Duroc boar semen quality across all parameters. The combination of extracellular (trehalose) and intracellular (glycerol) cryoprotectants provided superior protection compared to extenders without supplementation. BTS outperformed Tris-based extenders, likely due to its buffer composition. Despite this, a 45–47% decline in motility occurred during the freeze–thaw process, mainly due to cold shock-induced membrane damage and increased abnormalities. Although BTS achieved the highest post-thaw motility (25.88 ± 0.72%), it did not meet the SNI minimum standard of 30% progressive motility.

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