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Effects of Trehalose and Tris-Aminomethane Egg Yolk Extenders on Semen Quality of Duroc Boars Across Cooling, Before Freezing, and Post-Thawing Conditions

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Abstract:

This study aimed to evaluate the effectiveness of various semen extenders in preserving boar sperm quality during cryopreservation, with a focus on the use of trehalose as a cryoprotectant. Semen samples were collected from a Duroc boar and subjected to three treatment groups: T0 (BTS extender), T1 (BTS + 100 mM trehalose), and T2 (Tris-egg yolk extender). Semen was assessed at three stages: after cooling, before freezing, and post-thawing. The results showed that T1 significantly outperformed T0 and T2 across all parameters—maintaining the highest sperm motility and viability, and the lowest abnormalities after thawing. These findings indicate that trehalose effectively stabilizes sperm membranes and reduces cryoinjury. Therefore, the use of trehalose-enriched BTS extender is recommended for improving post-thaw semen quality in boars. Further research should explore field fertility outcomes and optimal trehalose concentrations.

Keywords — **Boar semen, Beltsville Thawing Solution (BTS), cryopreservation, sperm quality, Trehalose**

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I. INTRODUCTION

The swine industry in Indonesia plays a crucial role not only in meeting the rising demand for pork but also in maintaining cultural and traditional practices across various regions. The national pig population was estimated at 7.81 million heads in 2015 and showed growth until 2019. However, this number declined in 2020 due to the African Swine Fever (ASF) outbreak, before recovering to 8.01 million heads in 2021 [1]. Artificial Insemination (AI) technology has become an essential tool in pig breeding, allowing for the dissemination of superior genetic traits by introducing semen from genetically elite boars into sows through precise reproductive techniques [2].

While fresh liquid semen is commonly used in AI programs, frozen semen provides significant benefits such as extended storage time, improved management of breeding programs, and the potential to distribute genetics globally [3]. Furthermore, frozen semen contributes to the

preservation of genetic diversity, especially for endangered or rare pig breeds [4]. However, boar sperm cells are particularly vulnerable to damage caused by cold shock during freezing, primarily due to their high phospholipid and polyunsaturated fatty acid content in the plasma membrane [5].

Choosing an effective extender and cryoprotectant combination is critical to reducing cryoinjury and preserving semen quality after thawing. Beltsville Thawing Solution (BTS) is a widely accepted commercial extender known to maintain boar semen viability for short-term storage up to three days [6]. Cryoprotectants such as glutathione have demonstrated efficacy in preserving sperm motility after freezing and thawing [7]. Trehalose serves as an extracellular cryoprotectant, while glycerol protects intracellular structures during the cryopreservation process [8].

Supplementation of extenders with cryoprotective agents has been shown to enhance sperm survival by reducing cryoinjuries during freezing [9]. Additionally, sugars like trehalose play a role in osmotic balance and serve as an energy source to support sperm functionality during storage [10]. Therefore, refining extender compositions and cryoprotectant protocols is fundamental to improving the post-thaw quality of boar semen.

II. MATERIALS AND METHOD

A. Research Period and Location

This experimental study was carried out between October and December 2024 at the Regional Artificial Insemination Center, Baturiti, Tabanan Regency, Bali Province, Indonesia.

B. Research Materials

The semen samples were obtained from a single Duroc boar aged 2.5 years (ID: CAPOH 02; 240 kg). The animal received a daily ration of 4 kg concentrate feed, with unrestricted access to water. Semen was collected twice weekly using a dummy sow. A total of 10 ejaculates were collected, and only those with sperm motility exceeding 80% were processed for cryopreservation.

C. Research Method

A Completely Randomized Design (CRD) was employed with three treatment groups and ten replications each, totaling 30 samples. The treatment groups included:

- T0: BTS extender (control)
- T1: BTS extender supplemented with 100 mM trehalose
- T2: Tris-Aminomethane Egg Yolk extender without trehalose

D. Preparation of Egg Yolk

Fresh chicken eggs from commercial sources were used to prepare the egg yolk solution. The eggshells were disinfected with alcohol prior to cracking. The yolk was carefully separated from the albumen and placed on filter paper to absorb residual egg white. The cleaned yolk was measured in a graduated cylinder and diluted with distilled water in a ratio of 1:2 (15 mL yolk to 30 mL water). The mixture was homogenized for 15 minutes and centrifuged at 2,000 rpm for 15 minutes. The supernatant was collected to be used as clarified egg yolk.

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

The BTS powder (Minitub, Germany) was dissolved at 2.5 grams per 50 mL of distilled water and mixed thoroughly. Clarified egg yolk was added to the BTS solution in a 1:4 ratio (10 mL egg yolk to 40 mL BTS), and the combined extender was homogenized for 15 minutes. Before use, the extender was warmed to 37°C using a water bath.

F. Preparation of Tris-Aminomethane-Based Extender

The Tris extender was formulated by dissolving 1.82% Tris (hydroxymethyl aminomethane), 0.98% citric acid, and 0.25% fructose in 50 mL distilled water. Clarified egg yolk was added to the Tris solution at a 1:4 ratio and homogenized for 15 minutes. The mixture was warmed to 37°C and supplemented with 0.8 mL penicillin and 0.7 mL streptomycin as antibiotics, then homogenized for an additional 10 minutes.

G. Semen Processing and Freezing with BTS Extender

Fresh semen samples with motility above 80% were diluted with BTS–egg yolk extender at a 1:1 ratio. The diluted semen was held at room temperature (20–22°C) for 2 hours, then centrifuged at 2,000 rpm for 15 minutes to separate the sperm pellet from the supernatant.

H. Semen Processing and Freezing with Tris Extender

Semen was initially diluted 1:1 with Tris–egg yolk extender and equilibrated at room temperature for 2 hours. After centrifugation at 2,000 rpm for 15 minutes, the pellet was resuspended in extender containing 100 mM trehalose and 4% glycerol as cryoprotectants. This preparation was equilibrated at 5°C for 2 hours before packaging into 0.5 mL straws, each containing 200 million spermatozoa. Straws were placed on a freezing rack 5 cm above liquid nitrogen vapor for 20 minutes in a Styrofoam container, followed by immersion in liquid nitrogen for long-term storage.

I. Semen Quality Evaluation

Semen quality parameters assessed included sperm motility, viability, and abnormalities. **Motility**: Motility was observed under a light microscope, assessing the percentage of progressively motile spermatozoa. **Viability**: Eosin-nigrosin staining differentiated live (unstained) from dead (stained) sperm cells. **Abnormalities**: Sperm morphology was examined microscopically, noting defects such as detached or double heads, bent or broken tails, coiled, or bifurcated tails.

J. Data Analysis

The data obtained in this study were analyzed using Multivariate Analysis of Variance (MANOVA) to simultaneously examine the effects of treatments on multiple semen quality parameters, including motility, viability, and abnormalities. All statistical analyses were performed using R Studio software. When significant differences were detected ($p < 0.05$), post hoc tests were conducted to identify specific differences between treatment groups.

III. RESULTS AND DISCUSSION

The assessment of fresh semen quality was performed to confirm its suitability for subsequent processing and cryopreservation procedures. The average volume of the collected fresh semen from Duroc boars was 142.0 mL with a standard deviation of ±15.5 mL. This volume is lower compared to the range of 240 to 320 mL reported by [11], but it closely aligns with the average volume of 167.5 ± 19.84 mL documented by [12]. Variations in semen volume and other macroscopic traits can be influenced by factors such as the age of the boar, ejaculation frequency, and the degree of sexual stimulation during semen collection.

The fresh semen exhibited a characteristic milky-white color, with a pH value consistently measured at 7.8. The odor was typical for boar semen, indicating good sample integrity. Motility was observed at an average of 79.5% ± 1.58, suggesting a high proportion of progressively motile spermatozoa, while viability was recorded at 81.23% ± 0.59, confirming that most sperm cells were alive at the time of collection. The sperm concentration was measured at 272.3 ± 15.83 million cells per milliliter, demonstrating a robust sperm density adequate for cryopreservation. The percentage of sperm abnormalities was relatively low at 5.72% ± 0.22, indicating good structural integrity of sperm cells in the fresh semen samples.

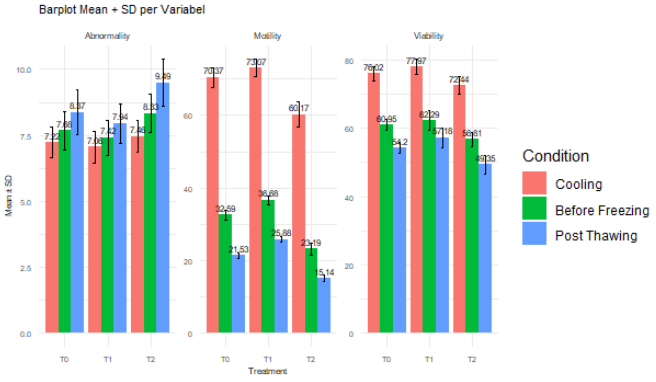

Fig. 1 Mean and Standard Deviation of Sperm Quality Parameters (Motility, Viability, Abnormality) Across Treatments and Conditions Cooling, Before Freezing, and Post Thawing).

The multivariate analysis of variance (MANOVA) indicated that both Treatment and Condition had a statistically significant effect on the combined semen quality parameters—motility, viability, and

abnormality ($p < 0.001$). Furthermore, a significant Treatment × Condition interaction was observed ($p = 0.008$), suggesting that the effect of extender formulations varied depending on the stage of semen handling and preservation (Cooling, Before Freezing, and Post Thawing).

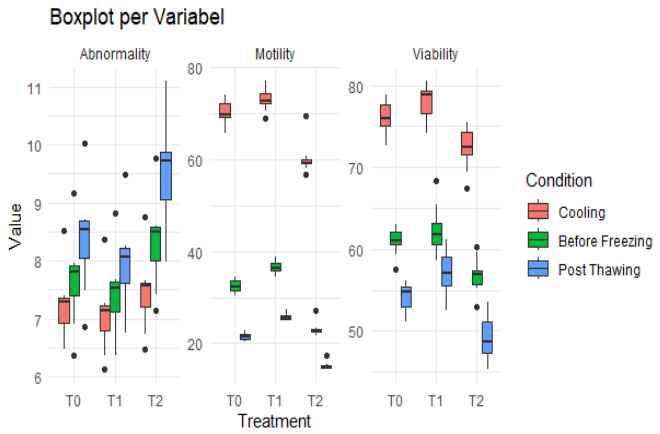

Fig. 2 Illustrates the distribution of sperm quality parameters—including abnormality, motility, and viability—across three treatment groups (T0, T1, T2) and three cryopreservation-related conditions (Cooling, Before Freezing, and Post Thawing).

Sperm Motility

Sperm motility was significantly influenced by Treatment, Condition, and their interaction, emphasizing the differential protective effects of the extenders at various stages of sperm processing. As shown in the boxplot (Fig.2), the Cooling condition preserved the highest motility values across all treatments, particularly in T0 and T1, where medians exceeded 70%. However, a marked decline in motility occurred following cryopreservation (Post Thawing), with T2 showing the most severe drop, falling below 20%, while T1 retained comparatively higher post-thaw motility values.

The T1 treatment, comprising BTS (Beltsville Thawing Solution) supplemented with 100 mM trehalose, consistently outperformed both T0 (BTS control) and T2 (Tris-egg yolk without trehalose), especially in the Post Thawing phase. Trehalose is known for its membrane-stabilizing and cryoprotective properties through water replacement and vitrification mechanisms, which contribute to maintaining motility even under freeze-thaw stress.

Sperm Viability

Sperm viability was also significantly affected by both Treatment and Condition, with the boxplot showing a parallel trend to motility. During the Cooling and Before Freezing stages, T1 maintained the highest viability, with median values around 75–80%. In contrast, T2 showed the lowest post-thaw viability, indicating a lack of effective cryoprotection. The consistent performance of T1 suggests that trehalose enhances membrane stability during both cooling and cryopreservation, thereby supporting sperm survival. In contrast, the Tris-egg yolk extender (T2) without trehalose failed to adequately protect spermatozoa from freezing-induced damage, possibly due to egg yolk's variable emulsification and lower osmotic buffering capacity.

Sperm Abnormality

Sperm abnormalities were influenced significantly by both Treatment and Condition, although no significant interaction was detected, suggesting that both factors independently contributed to increases in morphological defects. As illustrated in the boxplot, T2 consistently exhibited the highest abnormality rates, especially in the Post Thawing condition (above 9%), while T1 maintained the lowest rates across all processing stages. Cryoinjury, resulting from ice crystal formation, oxidative stress, and osmotic fluctuations, is a well-established cause of morphological defects, particularly in the tail and midpiece regions. The lower abnormality rates in T1-treated samples indicate more effective mitigation of these damaging processes, likely due to trehalose's ability to stabilize lipid bilayers and protect protein structures during dehydration and rehydration phases. Moreover, the addition of trehalose and glycerol to a BTS–egg yolk extender has been shown to enhance its ability to protect spermatozoa from cryogenic damage, compared to extenders that do not contain these supplements [13].

Semen samples were diluted using various types of extenders and initially kept at ambient temperature (20–22°C) to facilitate a gradual transition to lower temperatures before the freezing process commenced. The equilibration phase at 5°C serves as a pivotal step, during which sperm cells undergo physiological adaptation to the extender constituents, particularly cryoprotective agents such as trehalose and glycerol [14]. A substantial decline in motility was evident following the thawing procedure, with reductions observed in the range of 45–47%. This finding aligns with previous reports of considerable variation in post-thaw motility of boar semen, including values such as 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]. Thawing, typically carried out at 37°C for 20 seconds, has been shown to exert detrimental effects on the sperm plasma membrane. Given the membrane's critical role in regulating ion exchange and maintaining motility, any compromise in its structural integrity can severely impair sperm function. Indeed, damage sustained during the thawing process has been reported to affect approximately 40–50% of spermatozoa [18].

A significant decline in sperm viability was observed following the thawing process, with treatment T1 maintaining the highest viability level post-thaw, whereas T2 exhibited the. These findings indicate that T1 is the most effective treatment in preserving sperm viability throughout the cryopreservation cycle. The observed decrease in viability after thawing is plausibly attributed to structural alterations in the sperm plasma membrane induced by the freezing process. Such alterations impair membrane integrity and functionality, particularly its role in cellular protection, leading to diminished sperm viability [19].

The pattern of temperature shifts during cryopreservation—beginning with equilibration and continuing through to post-thaw handling—appears to significantly influence the structural and functional stability of spermatozoa. In this context, the cryopreservation medium used in T1, which includes BTS® extender, demonstrated superior efficacy in maintaining sperm viability. The BTS® formulation comprises glucose (as an energy substrate), EDTA (for calcium regulation), antibiotics (penicillin and streptomycin) to prevent microbial contamination, and buffering agents such as sodium citrate and sodium bicarbonate, along with potassium chloride which plays a role in reducing sperm motility to mitigate stress [20].

Moreover, the inclusion of trehalose, a disaccharide sugar, contributed to enhanced cryoprotective efficiency. Its superior performance compared to monosaccharides has been widely reported, particularly in stabilizing cellular membranes and proteins during freezing, thereby improving post-thaw sperm viability [20].

The detrimental effects observed during freezing and thawing are closely related to oxidative stress, which is intensified by the excessive generation of reactive oxygen species (ROS). These reactive species can compromise sperm function by inducing DNA fragmentation and other subcellular damages [21].

Furthermore, spermatozoa abnormalities were least prevalent in fresh semen and significantly increased after thawing. This trend underscores the damaging impact of both cold storage (at 5°C) and the freezing-thawing cycle on sperm morphology. The magnitude of temperature fluctuation appears to correlate positively with the extent of morphological defects. One of the primary contributing factors is cold shock, which disrupts the lipid bilayer of the sperm membrane and causes osmotic imbalance. Such disruptions may lead to physical anomalies, including detached or fractured sperm heads and tails [22,23]. These morphological alterations are consistent with the literature, where cold-induced structural stress is a major cause of sperm damage during cryopreservation [24,25].

IV. CONCLUSIONS

The study demonstrated that the use of BTS extender supplemented with 100 mM trehalose effectively preserved boar sperm quality during the cryopreservation process. T1 consistently showed

the highest post-thaw motility and viability and the lowest morphological abnormalities compared to T0 and T2. These findings suggest that trehalose enhances membrane stability and offers superior cryoprotective effects. Future studies are recommended to optimize trehalose concentration and explore its combination with other cryoprotectants. Additionally, field trials evaluating the fertility outcomes of post-thaw semen using T1 should be conducted to validate its practical application in artificial insemination programs.

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