



**SPERM LONGEVITY AND RECOVERY RATE OF BELGIAN BLUE CATTLE
IN DIFFERENT EXTENDER**

**Longivitas dan Recovery Rate Sperma Sapi Belgian Blue
dalam Berbagai Bahan Pengencer**

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ABSTRACT

Frozen semen from Belgian Blue cattle is in high demand among farmers, as evidenced by the increasing interest in this breed's semen. However, the freezing process reduces sperm motility and viability. This study aimed to assess the viability of sperm post-freezing and the sperm recovery rate (RR) in Belgian Blue cattle using different extenders. Semen was collected, evaluated, and diluted in four extenders: three commercially available ones—BoviFree[®], AndroMed[®], and Steridyl[®]—and a homemade extender, Tris Egg Yolk (TEY). Longevity and RR tests were conducted 24 h after freezing. Progressive motility was assessed using a portable computer-assisted sperm analysis (CASA) device (Androscope Minitube, Germany). The results indicated that sperm motility was higher in the BoviFree[®] and AndroMed[®] extenders after 3 h compared to the other extenders ($p < 0.05$). After 4 h, motility remained higher in the BoviFree[®] extender than in TEY. The RR values showed no significant differences among the extenders ($p < 0.05$). The study concluded that sperm longevity in Belgian Blue cattle was optimal in the BoviFree[®] extender, with consistently good sperm RR values across all extenders.

Keywords: *Commercial extender, Belgian blue, Frozen semen, Sperm longevity, Sperm recovery rate*

ABSTRAK

Semen beku sapi Belgian Blue sangat diminati oleh peternak. Tingginya minat peternak dibuktikan dengan meningkatnya permintaan semen beku rumpun sapi tersebut. Proses pembekuan menyebabkan penurunan motilitas dan daya tahan hidup sperma. rendah. Penelitian ini bertujuan untuk menguji ketahanan sperma (longivitas) setelah pembekuan serta daya tahan sperma terhadap pembekuan atau *recovery rate* (RR) pada sapi Belgian blue dalam berbagai bahan pengencer. Semen dikoleksi, dievaluasi dan diencerkan dalam empat bahan pengencer (Pengencer komersial: BoviFree[®], AndroMed[®] dan Steridyl[®]) serta pengencer home-made Tris Kuning telur (TKT)). Pengujian longivitas dan RR dilakukan 24 jam setelah pembekuan. Metode pengujian motilitas progresif menggunakan *Computer Assisted Sperm Analysis* (CASA) *portable* (Androscope Minitube, Germany). Hasil menunjukkan nilai motility sperma dalam pengencer Bovifree[®] dan AndroMed[®] lebih tinggi pada jam ke-3 dibandingkan pengencer lainnya ($p < 0,05$). Jam ke 4 motilitas dalam pengencer BoviFree[®] lebih tinggi dari TKT. Nilai RR antar pengencer tidak berbeda ($p > 0,05$). Penelitian ini menyimpulkan longivitas

sperma sapi Belgian blue paling baik dalam pengencer BoviFree® dan nilai RR sperma dalam semua pengencer sama baiknya.

Kata kunci: *Belgian blue, Longivitas sperma, Pengencer komersial, Recovery rate sperma, Tris kuning telur, Semen beku*

INTRODUCTION

Belgian Blue cattle are a breed of *Bos taurus* cattle originating from Belgium and have been developed since the mid-19th century. It has a high carcass percentage, reaching around 80%, making Belgian Blue known as an efficient beef cattle breed (Aditya et al. 2024). Although this breed has high beef production performance, it is known to have limited adaptability to tropical environments. This condition can affect semen quality, especially plasma membrane stability and sperm motility during the cryopreservation process.

The semen cryopreservation process is a critical stage in frozen semen production because it causes structural and functional damage to sperm (Ugur et al. 2019). One of the frozen semen quality requirements in SNI 4869-1:2024 is that semen must have a minimum post-thawing sperm motility of 40%. The motility of fresh semen to be frozen must be > 70%. The motility of fresh semen varies between cattle breeds, as does the resistance of sperm to freezing. Therefore, semen with sperm motility < 70% but with high resistance to freezing can still be frozen. The ability of sperm to survive freezing (freezability) can be assessed from the recovery rate value. The recovery rate (RR) is calculated by dividing the motility of frozen semen by the motility of fresh semen multiplied by 100%. The minimum RR value is 60%. The RR value varies between breeds, depending on the individual, breed of cattle, and extender used (Sukirman et al. 2020; Indriastuti et al. 2020).

Semen extenders are crucial for preserving sperm viability, motility, and membrane integrity (Baharun et al. 2017). They help maintain pH stability and sperm metabolism during and after freezing, inhibit bacterial growth, and minimize temperature-induced damage (Sharafi et al. 2022). An effective extender should maintain a pH

between 6.8 and 7.2, supply an energy source, include antioxidants to mitigate oxidative stress, and contain cryoprotective agents to shield the sperm plasma membrane from damage (Bustani and Baiee 2021). Frozen semen extenders can be homemade, such as egg yolk skim, egg yolk citrate, and Tris-egg yolk (Arif et al., 2022), or commercial, like AndroMed®, BoviFree®, and Steridyl® (Amal et al. 2019; Tahar et al. 2022; Riwu et al. 2023).

A commonly used homemade extender is the Tris-egg yolk-glycerol solution, which contains lecithin, phospholipids, and cholesterol from egg yolk, all contributing to sperm membrane stability. Commercial extenders like AndroMed®, BoviFree®, and Steridyl® are formulated with non-animal lecithin and soluble protein components that protect the plasma membrane during cooling and freezing. The biochemical differences among these extenders are believed to affect sperm motility post-freezing. The success of semen cryopreservation is evaluated based on sperm motility after thawing and other variables.

Post-thaw, sperm must remain viable long enough to be transported within the female reproductive organs, undergo capacitation, and fertilize the egg. Sperm viability after thawing is assessed through sperm longevity, known as the thermal resistance test (Solís et al. 2024). These are additional variables in periodic frozen semen quality testing as outlined in the Indonesian frozen semen production manual (Arifiantini et al. 2024). Arifiantini et al. (2005) found that frozen semen from Frisian Holstein (FH) cattle in a commercial soybean lecithin-based extender exhibited higher RR values and longer sperm longevity compared to a tris extender with egg yolk lecithin. This study aimed to analyze sperm longevity and calculate the RR value of frozen Belgian Blue bull semen in various extenders.

MATERIALS AND METHODS

The research procedure was approved by the IPB University Animal Ethics Committee (number 315-2025 IPB).

Place and time of research

Fresh semen was collected and frozen at the Artificial Insemination Center (BIB) in Lembang. Post-freezing observations were conducted at the Reproductive Rehabilitation Unit (URR) of the School of Veterinary Medicine and Biomedicine, IPB University. The research was conducted from 22 July to 2 September 2025.

Preparation of Semen Extenders

This study utilized four types of extenders: homemade Tris Egg Yolk (TEY) and three commercial extenders, namely, AndroMed[®], BoviFree[®], and Steridyl[®]. The TEY extender was composed of Tris buffer, which included Tris hydroxymethyl aminomethane (2.42 g), citric acid (1.28 g), and fructose (2.16 g), all dissolved in distilled water to make a total volume of 100 ml, with 20% (v/v) egg yolk added to complete the extender. The mixture was centrifuged at 2500 RPM for 15 min to separate the large lipid molecules. The supernatant was then collected, and 8% glycerol (v/v) was incorporated as a cryoprotectant, along with the antibiotics Spectinomycin, Gentamicin, Lincomycin, and Tylosin (Minitube, Germany). Commercial extenders were prepared by diluting them with aquabides: AndroMed[®] and BoviFree[®] at a 1:4 ratio and Steridyl[®] at a 1:1.5 ratio, following the manufacturer's protocol (Minitube, Germany). These extenders were homogenized and maintained in a water bath at 32–34°C until use.

Semen Samples and processing

Two Belgian Blue bulls from the Lembang Artificial Insemination Center (AIC), namely Widodo (5 years old), Sagara (3 years old), Adiwilaga (2 years old), and Aneska (2 years old), served as sources for semen collection. Fresh semen was collected biweekly and evaluated for sperm motility and concentrations. Only semen samples with sperm motility exceeding 70% were processed further. The evaluated se-

men was divided into four tubes, each diluted with one of the following extenders: TEY, AndroMed[®], BoviFree[®], and Steridyl[®]. The diluted semen was then packaged, equilibrated, and frozen. For longevity testing and RR calculation, frozen semen was stored in liquid nitrogen containers.

Sperm longevity testing of Belgian Blue cattle in various extenders

Sperm longevity testing was conducted individually. Frozen semen straws were thawed at 37°C for 30 seconds. After removing both stoppers, the semen was transferred to a microtube and maintained at 37°C. Sperm motility was assessed at 0, 1, 2, 3, and 4 h using a portable computer-assisted sperm analysis (CASA) device (Androscope, Minitube, Germany). Testing was performed as described by Arifiantini et al. (2024).

Calculation of Recovery Rate (RR)

The RR value was calculated by dividing the motility of frozen semen by the motility of fresh semen and multiplying by 100%, as described by Garner and Hafez (2000).
RR (%)

$$= \frac{\text{Sperm motility of frozen} - \text{thawed semen}}{\text{Sperm motility of fresh semen}} \times 100\%$$

RESULTS

Sperm longevity of Belgian blue frozen semen in with different extenders

The results indicated that progressive sperm motility at 0, 1, and 2 hours did not differ significantly between extenders, with values ranging from 46.00 to 53.00%, 31.50 to 41.12%, and 16.75 to 29.75%, respectively ($p > 0.05$). Notable differences in sperm motility emerged only after 3 h, as progressive sperm motility decreased over time across the extenders. After 3 h, sperm motility in the AndroMed[®] and BoviFree[®] extenders was higher than in the TEY extender ($p < 0.05$). At the same time, sperm in the Steridyl[®] extender showed no significant difference compared to those in the AndroMed[®], BoviFree[®], or TEY extenders ($p > 0.05$). Longevity analysis was conducted up to 4 hours, where BoviFree[®] had the

highest value, while sperm in the TEY extender had the lowest value (Table 1). No differences in sperm motility were observed

between the AndroMed[®] and Steridyl[®], extenders or between the BoviFree[®] and TEY extenders.

Table 1 Sperm longevity of Belgian Blue cattle in various semen extenders

Post-Thawing Period (h)	Semen extender (Motility %)				p-value
	TEY	AndroMed [®]	BoviFree [®]	Steridyl [®]	
0	53.00±8.02	51.25±5.01	46.00±7.44	47.00±6.53	0.87
1	31.50±6.86	41.12±4.70	33.75±6.04	37.62±6.30	0.75
2	16.75±5.35	29.75±4.62	27.75±5.19	27.00±6.08	0.33
3	7.00±2.76 ^b	23.37±4.42 ^a	25.25±3.82 ^a	18.12±4.47 ^{ab}	0.03
4	2.87±0.85 ^c	16.37±4.11 ^b	24.62±5.53 ^a	9.87±2.44 ^{bc}	0.00

Note: TEY, Tris Egg Yolk. The data are shown as mean ± standard error of the mean (SEM).

Different superscript letters following numbers in the same row indicate significant differences ($p < 0.05$).

Sperm Recovery rate of Belgian Blue frozen semen in different extenders

The results showed that sperm recovery rate did not differ between the extenders

($p > 0.05$). The recovery rate ranged between $57.12 \pm 8.20\%$ and $64.12 \pm 4.31\%$ (Table 2).

Table 2 Sperm Recovery Rate (RR) of Belgian blue frozen semen in different extenders

Extender	Progressive Motility (%)		Recovery rate
	Semen Segar	Post Thawing	
TEY		53.00±8.02	65.75±8.54
AndroMed [®]	79.25±5.06	51.25±5.01	64.12±4.31
BoviFree [®]		46.00±7.44	57.12±8.20
Steridyl [®]		47.00±6.53	58.37±6.47
p-value		0.87	0.78

Note: TEY = Tris Egg Yolk. The data shown as means ± standard error of the mean (SEM).

Different superscript letters following numbers on the same row indicate significant differences ($p < 0.05$).

Cryopreservation involves storing cells or tissues at extremely low temperatures, significantly reducing or even halting their metabolic activity. This method is frequently employed for the long-term preservation of genetic material and plays a crucial role in animal reproduction and conservation programs (Ananda et al. 2025). Despite its many benefits, cryopreservation can cause structural and functional damage to sperm during the freezing and thawing processes, affecting motility, viability, and plasma membrane integrity. The formation of ice crystals, along with osmotic and oxidative stress, disrupts sperm function and diminishes fertility (Xu et al. 2025). This process results in various forms of damage to the anatomical and physiological structure and function of cells, ultimately reducing their functionality (Sharafi et al. 2022). Additionally,

cryopreservation can alter the kinetic parameters that describe sperm motility patterns, thereby decreasing overall quality (Mokhtassi-Bidgoli et al. 2023).

In Indonesia, sperm extenders used in artificial insemination centers (AICs) are often either homemade or commercially sourced (Arif et al. 2022). Homemade versions typically include animal-based ingredients like egg yolk and milk. For a long time, extender made with whole or skim milk have served as semen preservatives (Miguel-Jimenez et al. 2020). Currently, AndroMed[®], a lecithin-based extender derived from soybeans, is the sole commercial extender employed in some AICs. Using dilution agents that incorporate plant-based or synthetic proteins, such as liposomes to replace animal lipoproteins, can help mitigate the risk of diseases linked to animal

products and enhance biosafety by curbing disease transmission (Bustani and Baiee 2021).

Egg yolk has long been utilized as a non-permeable cryoprotectant in tris-based extenders, offering protection to sperm by preventing thermal shock, sustaining motility, and preserving acrosome and mitochondrial integrity. However, soy lecithin- or phospholipid-rich liposomes are considered safer and more effective alternatives (Miguel-Jimenez et al. 2020). Maintaining mitochondrial structure is vital, as this organelle is central to ensuring sperm viability.

Studies on longevity and Thermal Resistance Test (TRT) are still rare in Indonesia. Solís *et al.* (2024) reported that TRT for 0.5, 1, and 2 hours was higher in Jersey cattle than in Holstein cattle. Arifiantini *et al.* (2005) reported that the longevity of FH bull semen in AndroMed[®] extender was 10% after 6 hours of storage. This study showed that BoviFree[®] survived for more than 20% after 4 h of incubation. The reduction in sperm motility during the first hour of incubation was 10–13% for commercial extenders and 21.5% for TEY. The reduction after 2 and 3 hours was 6–11% and 2.5–8.88% in commercial extenders and 14% and 9.75% in TEY extenders, respectively. Sperm in the BoviFree[®] extender showed the least reduction in motility. BoviFree[®] is a commercially available extender containing synthetic liposomes. The mechanism by which synthetic liposomes protect the cell membranes of Belgian Blue cattle sperm and enable the mitochondria to produce energy more efficiently than other extenders is not yet known, but it is suspected that there is compatibility between the extender and the cattle breed (Sukirman et al. 2020).

Sperm require a consistent energy source to sustain cellular functions essential for their survival and ability to fertilize. This energy is derived from external nutrients, which are converted into adenosine triphosphate (ATP), the main energy provider for various cellular processes. ATP is generated through oxidative phosphorylation within the mitochondria, and the ATP produced is then delivered to the microtubules to facilitate the movement of flagella, which is crucial for sperm motility. In bovine sperm,

the ATP level is closely linked to both motility and the potential for fertilization (Matatine et al. 2014).

Mitochondria play a crucial role not only in energy production but also in maintaining cellular redox equilibrium and controlling apoptosis. Nevertheless, oxidative phosphorylation (OXPHOS) generates reactive oxygen species (ROS) as natural by-product, thereby connecting energy metabolism to the management of oxidative stress (Xu et al. 2025). Consequently, sperm extender formulations that can balance mitochondrial function with ROS regulation are essential for preserving the quality of frozen-thawed bovine semen.

Table 2 indicates that recovery rates did not significantly differ among the extenders. This suggests that the TEY extender is as effective as commercially available options. According to the Indonesian standard (SNI) for frozen bovine semen No. 4869-1:2024, fresh semen must have a progressive motility of at least 70% to be processed into frozen semen. While local cattle sometimes exhibit sperm motility below this standard, they can still be processed into frozen semen if they demonstrate high freezeability. A minimum recovery rate of 60% (BSN 2024) and sperm motility of less than 70% are acceptable. The study results showed that only Belgian Blue cattle diluted with TEY and AndroMed[®] extenders achieved RR values above 60%.

Semen in TEY extender, although it has an RR value > 60%, must be inseminated immediately as it decreases by 21.5% within one hour. Sperm motility in the AndroMed[®] extender decreased by only 10%. Therefore, artificial insemination with this sperm can be performed 1 h after thawing. Sperm in the TEY extender had the highest RR percentage compared to the other extenders. This may be attributed to the egg yolk and 8% glycerol content in the extender. The glycerol content of 8% is considered effective for protecting cells during freezing, but it must be borne in mind that cryoprotectants are also toxic to sperm. Glycerol is an important component of cryopreservation because of its ability to prevent cell damage caused by freezing. Glycerol hydroxyl groups can inhibit the formation of

ice crystals that damage cell membranes (Hanifah et al. 2020).

Glycerol is capable of crossing cell membranes and affecting water molecules, which helps control the rate and degree of cellular dehydration during membrane phase changes induced by freezing. This process involves forming hydrogen bonds with water and creating amorphous solids at extremely low temperatures, known as vitrification. Cells preserved with glycerol exhibit excellent vitality upon thawing, making glycerol a key component in sperm extenders to preserve sperm quality and motility (Larasati et al. 2022). Sperm in TEY extenders tend to have a shorter lifespan, likely due to the high glycerol concentration. High glycerol concentration is toxic to the sperm cell. According to Sharafi et al. (2022), glycerol affects the components of the plasma membrane, including the glycocalyx, glycoproteins, and glycolipids, potentially causing membrane damage and increasing the viscosity of the cytosol. In contrast, commercial extenders contain only 6% glycerol, which is believed to result in a longer lifespan.

CONCLUSION

BoviFree® extender demonstrated the highest sperm longevity in Belgian Blue frozen semen, particularly evident after 3–4 hours of incubation, where it showed a slower reduction in progressive motility compared to other extenders. In contrast, TEY extender exhibited the fastest decrease in motility, especially within the first hour post-thawing. Despite these differences in longevity, sperm recovery rates did not significantly differ among extenders, ranging from 57.12% to 64.12%. The BoviFree® extender demonstrated greater sperm longevity for Belgian Blue bovine semen compared to other extenders. However, the sperm recovery rates remained consistent across the TEY extenders.

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