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Characteristics and Kinematics of Fresh Belgian Blue Semen and Its Resistance to Frozen–Thaw Using Different Extenders

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ABSTRACT

Extenders are used to preserve semen quality during storage. The Belgian Blue is a cattle breed known for its high dressing percentage; however, male reproductive performance is relatively low due to small testes size. This study aimed to analyze the characteristics and kinematics of fresh and frozen Belgian Blue semen. Fresh semen samples were collected from four Belgian Blue bulls, and their quality was assessed macroscopically, microscopically, and kinematically using portable computer-assisted sperm analysis (CASA) immediately after collection. Semen samples with progressive sperm motility ≥70% were diluted with the following extenders: Tris–egg yolk (TEY) and the commercial extenders AndroMed®, BoviFree®, and Steridyl®. CASA results showed that bulls differed significantly in ejaculate volume, color, consistency, sperm kinematics, including velocity curvilinear (VCL), velocity straight-line (VSL), and velocity average path (VAP), and sperm motility; however, none of these variables differed among frozen–thawed semen samples treated with the different extenders. In contrast, total motility, slow motility, and immotile sperm in frozen–thawed semen differed significantly between bulls. In conclusion, the characteristics of fresh Belgian Blue semen vary between individuals. Moreover, TEY, AndroMed®, BoviFree®, and Steridyl® showed comparable protective capabilities for frozen–thawed semen.

***Keywords:** Belgian Blue; cryopreservation; semen extender; frozen semen; sperm kinematics*

INTRODUCTION

Belgian Blue cattle are renowned for their significant muscular development due to a loss-of-function mutation in the myostatin gene (Meyermans *et al*., 2022). This breed is also recognized for its high carcass value, with dressing percentages ranging from 71.9% in cows to 75.54% in bulls (Fiems *et al*., 2003). Moreover, the breed is known for its remarkable growth characteristics; however, the flavor intensity of its meat is relatively low due to lower fat content compared with other breeds and its double-muscle conformation (Keady *et al*., 2017; Santos *et al*., 2021).

According to Hoflack *et al*. (2006), the relatively small testes of Belgian Blue cattle contribute to their low reproductive performance. The Ministry of Agriculture of Indonesia introduced Belgian Blue cattle under Decree No. 7919/OT.050/F2.2/01/2018, which also encouraged local farmers to implement crossbreeding programs with indigenous cattle, resulting in increased demand for frozen semen. The aim of such programs is to obtain superior progeny with improved growth rates and productivity (Aminurrahman *et al*., 2021). In Indonesia, the production and freezing of semen from Belgian Blue bulls are carried out at only two facilities: the Lembang Artificial Insemination Center and Singosari National Artificial Insemination Center.

During cryopreservation, semen undergoes extreme temperature shifts from approximately 34 °C to −196 °C, resulting in structural changes in sperm cells. In addition, the cryopreservation process leads to the formation of reactive oxygen species and a reduction in natural antioxidant levels (Arif *et al*., 2022). Semen extenders, which are essential for protecting sperm during freezing, are generally classified into two types: homemade and commercial. In both cases, they are designed to supply nutrients as energy sources for sperm, as well as lipoproteins and lecithin that protect sperm from cold shock and reduce oxidative metabolism (Tethool *et al*., 2022). Homemade extenders, such as tris–egg yolk (TEY) and skimmed milk, are commonly used at artificial insemination centers (AICs) because they are inexpensive and easily formulated using available materials. However, the effectiveness of homemade extenders may vary depending on the preparation procedure and the quality of the ingredients used. In contrast, commercial extenders are manufactured according to standardized formulations that include protective components. (Sukirman *et al*., 2019; Riwu *et al*., 2023).

Commercial extenders are industrially formulated and designed to maintain sperm viability using specific nutrients and functional components, including lecithin that is obtained from various sources. For example, AndroMed® and BioXcell® contain plant-based lecithin from soybeans, whereas Steridyl® contains animal-based lecithin extracted from egg yolk. Other commercial extenders such as BoviFree® and OptiXcell® contain liposomes (synthetic lecithin) and have been used as membrane protectants during the cryopreservation process (Riwu *et al*., 2023; Arifiantini *et al*., 2024).

The sperm membrane consists of a lipid bilayer composed of phospholipids, cholesterol, and various proteins (Pini *et al*., 2018). In mammals, the phospholipid composition of the plasma membrane is asymmetric, with phosphatidylcholine and sphingomyelin located mainly in the outer layer of the membrane, whereas phosphatidylserine and phosphatidylethanolamine are mainly concentrated in the inner layer. According to Castro *et al*. (2025), the plasma membrane of sperm from different species differs based on their respective cholesterol levels, which are mainly expressed in terms of the cholesterol-to-phospholipid ratio and the degree of fatty acid saturation in glycerophospholipids.

Breed-specific differences in the frozen–thaw tolerance of semen are associated with the composition of sperm plasma membrane. In addition, compatibility with semen extenders also varies among breeds (Sukirman *et al*., 2019). Currently, there is limited information on the characteristics of Belgian Blue semen and its resistance to the frozen–thaw process with different extenders. Therefore, this study aimed to evaluate the characteristics of fresh Belgian Blue semen, with a special focus on its frozen–thaw resistance when diluted with different extenders.

MATERIALS AND METHODS

Time and Location

This study was performed from July to September 2025. Fresh semen samples were collected and cryopreserved at the Lembang AIC. Post-thaw evaluations were performed at the Reproductive Rehabilitation Unit, School of Veterinary Medicine and Biomedical Sciences, IPB University. The research protocol was approved by the Animal Ethics Committee of IPB University (Approval No. 315-1.2025 IPB).

Animals

Four Belgian Blue bulls (Widodo, Sagara, Adiwilaga, and Aneska, aged 5, 3, 2, and 2 years, respectively) were maintained under uniform feeding and housing conditions. Each bull was housed individually in a 2.5 × 4 m pen with concrete flooring and bedding material, maintained under ambient temperature ranging from 28–34 °C, relative humidity 60%–90%, with natural lighting and ventilation. Bulls were fed a total mixed ration twice daily and fresh water was available *ad libitum*.

Preparation of Semen Extenders

The four semen extenders tested were a homemade TEY extender and the commercial extenders AndroMed®, BoviFree®, and Steridyl® (all obtained from Minitube, Germany). The TEY extender consisted of tris(hydroxymethyl)aminomethane (2.42 g), citric acid (1.28 g), and fructose (2.16 g), all dissolved in distilled water to a final volume of 100 mL. After adding 20% (v/v) hen egg yolk to complete the extender formulation, we centrifuged the mixture at 2500 rpm for 15 min to remove lipids. The resulting supernatant was then supplemented with 8% (v/v) glycerol (as a cryoprotectant) and antibiotics (spectinomycin 600 µg, gentamycin 500 µg, lincomycin 300 µg, and tylosin 100 µg; Minitube, Germany) (Arifiantini *et al*., 2024).

Collection and Evaluation of Fresh Semen

Semen was collected twice a week in the morning using an artificial vagina, following the standard operating procedure of Lembang AIC. Semen quality was evaluated macroscopically and microscopically immediately after collection.

Macroscopic characterization of semen evaluated semen volume, color, consistency, and pH. Color was scored as 1 (yellow), 2 (milky), or 3 (creamy), while consistency was scored as 1 (watery), 2 (moderate), or 3 (thick).

Microscopic characterization of semen assessed motility, viability, acrosomal integrity, and sperm morphology. Sperm motility and kinematic variables were analyzed using portable computer-assisted semen analysis (AndroScope, Minitube, Germany). Analysis was performed using a 3 µL semen aliquot placed in a prewarmed (37 °C) counting chamber (Minitube, Germany). Four microscopic fields were examined at 200× magnification, with approximately 300 sperm analyzed per sample. Acrosomal integrity was determined by preparing a semen smear on a glass slide, followed by air-drying, fixation with 95% ethanol for 10 min, and staining with Giemsa (Merck, Germany) for 2.5 h. Stained samples were examined at 400× magnification using a compound light microscope (Olympus CX 23, Japan) (Chowdhury *et al*., 2014).

Frozen Semen Processing

Fresh semen samples with sperm motility ≥70% were selected for freezing. Each sample was divided into four subsamples and diluted with TEY, AndroMed®, BoviFree®, and Steridyl® extenders to a sperm concentration of approximately 25 million sperm per 0.25 mL, or 100 million sperm per mL. We filled 0.25 mL Minitube straws with the diluted semen, and these were allowed to equilibrate for 4 h at 4 °C, followed by freezing in nitrogen vapor (i.e., holding the straws 4 cm above a liquid nitrogen surface for 15 min). Frozen semen was immersed and stored in liquid nitrogen (Ramírez-Vasquez *et al*., 2019). Post-thaw evaluation was performed at the Reproductive Rehabilitation Unit, IPB University.

Frozen Semen Evaluation

Frozen semen straws were thawed at 37 °C for 30 s, after which the semen was transferred into a microcentrifuge tube. Thawed semen samples were maintained at 37 °C during evaluation. The following semen characteristics were evaluated: motility, kinematics, viability, acrosome integrity, and sperm abnormalities using the same procedures as described for fresh semen, with minor modifications (Baharun *et al*., 2025). Modification in the ratio of frozen semen sample with HOS solution or with eosin nigrosine.

Statistical Analysis

Data were analyzed using SPSS software (version 26; IBM Corp., Armonk, NY, USA). ANOVA was performed at a 95% confidence level, followed by Duncan's Multiple Range Test to evaluate the differences between treatments. Results are shown as mean ± standard error of mean.

RESULTS

Macroscopic and Microscopic Quality of Fresh Belgian Blue Semen

The differences in semen volume, color, and consistency between individual bulls were significant. Particularly, although the semen volumes of Widodo and Sagara did not differ significantly, both were higher than that of Adiwilaga. No significant differences were detected between the semen volumes of Sagara and Aneska or between Aneska and Adiwilaga. Overall, Belgian Blue bull semen volume ranged from 2.50 to 6.25 mL (Table 1). The semen color of Widodo and Aneska varied from yellowish white to milky white, whereas those of Sagara and Adiwilaga were milky white and milky white to creamy, respectively. Semen pH ranged from 6.61 to 6.67, with no significant differences observed between the bulls.

Sperm motility analysis of fresh Belgian Blue semen (Table 2) showed significant differences between bulls in several microscopic semen traits. Specifically, progressive motility was lower in sperm from Adiwilaga and Aneska than in sperm from Widodo. Furthermore, relatively more immotile sperm were observed in Sagara and Adiwilaga than in Widodo.

The results of kinematics analysis of fresh Belgian Blue semen (Table 3) show no significant differences in the following distance curvilinear line, distance straight line (DSL), distance average path (DAP), amplitude of lateral head (ALH), beat cross frequency (BCF), linearity (LIN), and straightness (STR). In contrast, the following traits varied significantly between bulls: curvilinear velocity (VCL), straight line velocity (VSL), and average path velocity (VAP). Widodo produced semen with the highest VCL compared to those of Sagara and Adiwilaga. The VCL values of Widodo and Adiwilaga did not differ significantly; however, both were higher than those of the other bulls. We observed the highest VAP in Widodo semen, although it did not differ significantly from that of Aneska, while the lowest values were observed in Sagara and Adiwilaga.

Quality of Belgian Blue Semen After Freezing with Different Extenders

Table 4 shows that the total and slow motility of sperm in TEY were significantly higher than those of AndroMed®, BoviFree®, and Steridyl®. The different extenders had no significant effect on any of the sperm kinematic parameters (Table 5).

Table 1. Macroscopic qualities of fresh Belgian Blue semen (n = 16)

Variables	Bull name (means±SE)				p-value
	Widodo	Sagara	Adiwilaga	Aneska	
Semen volume (mL)	6.25±0.83^{a}	4.75±0.25^{ab}	2.50±0.20^{c}	3.12±0.65^{bc}	0.00
Color	1.50±0.29^{c}	2.00±0.00^{b}	2.25±0.25^{a}	1.25±0.25^{c}	0.03
Consistency	1.75±0.25^{a}	1.00±0.00^{b}	1.25±0.25^{ab}	1.00±0.00^{b}	0.03
pH	6.60±0.10	6.68±0.10	6.75±0.08	6.66±0.11	0.78

Note: n = Number of ejaculate; SE = Standard error; Semen color = 1 (yellow), 2 (milky white), and 3 (cream); Consistency = 1 (watery), 2 (moderate), and 3 (thick). Different superscript letters within the same row indicate significant differences.

Table 2. Microscopic qualities of fresh Belgian Blue bull semen (n = 16)

Variables (%)	Bulls name (means ±SE)				p-value
	Widodo	Sagara	Adiwilaga	Aneska	
Total motility	96.45±0.17^{a}	81.67±2.42^{b}	77.82±7.13^{b}	84.87±3.00^{ab}	0.04
Progressive motility	93.63±0.44^{a}	76.85±1.89^{b}	70.83±7.83^{b}	79.10±4.16^{b}	0.02
Fast motility	75.89±1.62^{a}	53.07±1.62^{b}	50.32±6.90^{b}	54.76±5.74^{b}	0.01
Slow motility	5.69±0.87^{a}	15.38±2.62^{b}	13.17±1.74^{b}	13.50±2.88^{b}	0.03
Circle motility	12.05±1.43	8.27±2.04	6.80±1.09	10.84±2.24	0.19
Local motility	2.57±0.22^{b}	4.82±0.80^{ab}	6.99±0.78^{a}	5.77±1.32^{a}	0.02
Immotile	3.80±0.25^{b}	18.33±2.42^{a}	22.18±7.13^{a}	15.12±3.00^{ab}	0.04
Sperm viability	89.74±4.08	79.24±3.02	74.55±5.55	70.34±11.66	0.07
Sperm acrosomal integrity	96.99±0.61	92.01±1.73	87.65±5.05	79.06±4.00	0.10
Sperm Abnormality	4.83±1.46	7.48±1.70	11.09±2.05	12.39±3.24	0.12

Note: n = Number of ejaculate; SE = Standard error; Different superscript letters following the values in the same row indicate significant differences.

DISCUSSION

Different Belgian Blue bull individuals can produce significantly different volumes of ejaculate. This finding is consistent with that of Hoflack *et al*. (2006), who reported a mean semen volume of 5.52 ± 1.95 mL in Belgian Blue bulls. Semen color varied from yellowish white to milky white to creamy, which is related to riboflavin pigments that are regulated by autosomal recessive genes. At normal levels, riboflavin does not affect semen quality, and it may even act as a natural antioxidant (Hoesni *et al*., 2024).

Semen characteristics, including volume and color, can be influenced by age, with older bulls typically producing more semen due to greater testicular mass and activity of the accessory glands (Vince *et al*., 2018). The semen of younger bulls is usually more concentrated within a smaller ejaculate volume and exhibits greater sensitivity to elevated environmental temperatures (Taaffe *et al*., 2022).

The pH of bull semen normally ranges from 6.4 to 7.8 (Tethool *et al*., 2022), which was observed for all the bulls in this study, suggesting stable pH conditions and optimal sperm metabolism. A decrease in pH during

Table 3. Sperm kinematics of fresh Belgian Blue bull semen (n = 16)

Sperm kinematics	Bulls name (means ± SE)				p-value
	Widodo	Sagara	Adiwilaga	Aneska	
VCL (μm/s)	205.07±7.77^{a}	175.82±2.61^{b}	183.67±2.75^{b}	187.37±9.89^{ab}	0.04
VSL (μm/s)	89.05±4.70^{a}	68.06±5.77^{b}	85.02±5.85^{a}	68.79±1.82^{b}	0.02
VAP (μm/s)	106.34±4.01^{a}	83.77±4.58^{b}	88.01±2.38^{b}	96.99±0.96^{ab}	0.04
DCL (μm)	49.91±0.88	45.58±2.97	46.36±0.32	48.29±2.33	0.42
DSL (μm)	20.44±1.81	16.58±3.08	16.64±0.34	20.23±2.01	0.39
DAP (μm)	25.42±1.61	21.21±2.85	21.57±0.33	25.01±2.19	0.33
ALH (μm)	4.00±0.20	3.52±0.02	3.78±0.05	3.67±0.05	0.11
BCF (Hz)	15.65±1.19	15.00±1.31	13.98±0.65	15.83±0.81	0.54
LIN (VSL/VCL)	0.43±0.03	0.37±0.03	0.38±0.01	0.42±0.02	0.27
STR (VSL/VAP)	0.79±0.02	0.76 ±0.03	0.75±0.01	0.78±0.01	0.49

Note: n = Number of ejaculate; SE = Standard error; VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, DCL = distance curved line, DSL = distance straight line, DAP = distance average path, ALH = amplitude of lateral head, BCF = beat cross frequency, LIN = linearity, STR = straightness. Different superscript letters in the same row indicate significant differences.

Table 4. Post-thaw quality of frozen Belgian Blue bull semen treated with different extenders (n = 24)

Variables (%)	Bulls name (means ± SE)				p-value
	TEY	AndroMed®	BoviFree®	Steridyl®	
Total motility	72.49±4.88^{a}	54.12±3.70^{b}	54.60±5.28^{b}	56.75±4.44^{b}	0.03
Progressive motility	62.32±5.51	45.35±4.04	46.39±5.67	45.31±4.39	0.06
Fast motility	36.20±4.68	28.30±3.51	30.60±5.52	25.52±2.44	0.35
Slow motility	23.07±1.93^{a}	13.74±0.28^{b}	13.62±1.37^{b}	18.07±1.88^{b}	0.00
Circle motility	3.08±0.30	3.30±0.63	3.30±0.63	1.72±0.38	0.16
Local motility	10.17±1.32	8.78±0.73	7.98±1.11	11.44±0.63	0.10
Immotile	27.51±4.88^{b}	45.88±3.70^{a}	45.63±5.42^{a}	43.25±4.44^{a}	0.03
Sperm viability	66.63±4.18	59.27±3.87	60.53±4.07	62.02±2.46	0.54
Sperm acrosomal integrity	88.92±1.85	93.20±1.52	93.14±1.29	89.98±2.08	0.31
Sperm abnormality	8.76±0.61	7.03±0.96	6.93±1.12	8.40±1.14	0.46

Note: n = Number of straws analyzed; SE = Standard error; TEY = Tris–egg yolk. Different superscript letters following the values in the same row indicate significant differences.

Table 5. Post-thaw sperm kinematics of frozen Belgian Blue bull semen treated with different extenders (n = 24)

Variables	Extender (Means ± SE)				p-value
	TEY	AndroMed®	BoviFree®	Steridyl®	
VCL (μm/s)	122.16±6.33	141.10±4.97	130.27±8.47	118.82±4.23	0.08
VSL (μm/s)	49.58±2.93	57.51±3.87	50.43±4.01	52.40±3.11	0.40
VAP (μm/s)	59.24±3.27	68.37±3.33	60.60±4.56	60.49±2.97	0.28
DCL (μm)	38.30±1.14	42.78±1.82	40.58±1.98	36.82±0.81	0.06
DSL (μm)	15.15±0.71	17.01±1.82	15.31±0.97	15.92±0.63	0.66
DAP (μm)	18.40±0.64	20.52±1.62	18.63±1.10	18.62±0.48	0.47
ALH (μm)	2.74±018	3.01±0.13	2.91±0.17	2.48±0.09	0.09
BCF (Hz)	14.48±0.52	14.91±1.05	14.11±0.52	15.77±0.58	0.40
LIN (VSL/VCL)	0.47±0.05	0.38±0.03	0.39±0.01	0.41±0.00	0.36
STR (VSL/VAP)	0.82±0.03	0.76±0.03	0.77±0.01	0.78±0.01	0.55

Note: n = Number of straws analyzed; SE = Standard error; TEY = tris–egg yolk–glycerol; VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, DCL = distance curved line, DSL = distance straight line, DAP = distance average path, ALH = amplitude of lateral head, BCF = beat cross frequency, LIN = linearity, STR = straightness. Different superscript letters within the same row indicate significant differences.

storage can occur through lactic acid accumulation from fructose metabolism, which can negatively affect sperm motility (Dey *et al*., 2019).

The bull Widodo produced sperm with the highest total, progressive, and fast motility, indicating the highest sperm movement performance. There were no significant differences in circular motility variables between the bulls, indicating that circular motility is consistent across individuals and is not a major factor affecting overall motility. Higher progressive and fast motility values are associated with mitochondrial activity and more efficient flagellar activity (Bahmid *et al.,* 2023). According to the Indonesian National Standard for frozen bull semen, sperm must have a minimum progressive motility of 70% to qualify for cryopreservation.

An intact plasma membrane is required for sperm to penetrate the zona pellucida; thus, viability and membrane integrity are important indicators of fertilization potential (Pardede *et al*., 2020). Sperm viability is strongly related to motility because the latter is dependent on membrane integrity and stability (Rahman *et al*., 2011). We observed high viability values in the sperm of all bulls, indicating their metabolic activity and membrane functionality were maintained. Furthermore, all samples showed less than 20% abnormal sperm, complying with the Indonesian National Standard for semen suitable for freezing (BSN, 2024).

Widodo displayed consistently high values of VCL, VSL, and VAP, suggesting this bull produced faster moving sperm than the other bulls. High VCL, VSL, and VAP values reflect sperm with greater efficiency in reaching the oocyte and are therefore reliable indicators of sperm quality (Kathiravan *et al*., 2011). Morrell *et al*. (2018) reported that VAP, VCL, and VSL values are generally higher in dairy bulls, whereas STR and LIN values are higher in beef bulls. We observed no significant differences in total motility, progressive motility, and BCF among bulls. These characteristics may vary depending on physiological and genetic differences between beef and dairy breeds.

Contri *et al*. (2013) considered sperm with VAP ≥80 μm/s and STR ≥75% as progressively motile. All the bulls in this study exceeded these thresholds. Hyperactive sperm are characterized by VCL values above 70 μm/s and ALH values above 7 μm (Kathiravan *et al.,* 2011). Although Belgian Blue bulls showed VCL values >70 μm/s, ALH values ranged from 3.52 to 4.00 μm, indicating that none of the bulls produced sperms with hyperactivated motility.

Mitochondria are the main source of energy for sperm motility (Xu *et al*., 2025). Therefore, progressive motility depends on optimal mitochondrial function. Widodo showed the highest progressive and fast motility, and therefore, this bull has the greatest fertility potential. Our analysis of Belgian Blue bulls found no differences ($p>0.05$) in sperm viability, acrosome integrity, or sperm abnormalities among the four Belgian Blue bulls.

Semen extenders are crucial for preserving sperm membranes and their components, reducing cellular stress, and providing essential nutrients within a stable environment (Bustani & Baiee, 2021). These functions operate during the cooling and cryopreservation stages to minimize potential cellular damage. Sperm with the highest slow motility values were observed in semen diluted with TEY extender, while semen diluted in commercial extenders showed slow motility ranging from 13.62% to 18.07%. These findings indicate that TEY is more effective than the commercial extenders in maintaining the metabolic activity of Belgian Blue bull sperm after freezing and thawing. This result does not agree with that of Baharun *et al*. (2017), who reported that the total motility of sperm in Pasundan bulls is higher in semen extended with AndroMed® than in semen extended with TEY. We also observed relatively more immotile sperm in semen treated with commercial extenders (43.25%-45.88%) than in semen treated with the TEY extender (27.51%).

Immotility in sperm is an indication of low energy for movement. Nonviable sperm are incapable of fertilizing the oocyte and are characterized by a lack of movement as well as damage to their plasma membrane. Sperm motility depends on energy availability, and carbohydrates are the main energy source. TEY has a higher carbohydrate concentration, and semen treated with this extender had fewer immotile sperm. Carbohydrates provide energy for sperm metabolic pathways that support motility and viability (Rotimi *et al.*, 2024). The fructose present in extenders is metabolized via glycolysis and mitochondrial respiration to produce ATP that is used to support sperm motility and viability (Pappa *et al.,* 2019).

The glycerol concentrations in the commercial and TEY extenders were approximately 6.4% and 8%, respectively. Glycerol is a common cryoprotectant that protects sperm by replacing intracellular water molecules and stabilizing the plasma membrane shifts during freezing (Yendraliza & Rahman, 2023), and therefore, the higher glycerol concentration in the TEY extender may provide greater cellular protection than commercial extenders.

All semen extenders contain lecithin derived from various sources, such as egg yolk, milk, soy, and liposomes (Gunawan *et al.,* 2025). Egg yolk–derived lecithin in TEY and Steridyl® extenders plays a crucial role in maintaining sperm functionality during storage. Phosphatidylcholine (PC), which accounts for approximately 73% of the total phospholipid content in egg yolk lecithin, is essential for preserving plasma membrane stability and integrity, particularly during cooling and freezing. The lecithin concentration in egg yolk is approximately 10% of the total egg yolk phospholipids. The structural components of the sperm plasma membrane, including phospholipid PC, are crucial for preserving cellular stability and sperm function (Zhao *et al.*, 2023).

Soy lecithin in AndroMed® and synthetic lecithin in BoviFree® also stabilize the sperm membrane during cryopreservation, thereby supporting higher progressive motility. The synthetic liposomes in BoviFree® are the main components of the sperm plasma membrane and can replace phospholipids damaged during freezing, thereby preserving membrane integrity and sperm viability (Nsairat *et al*., 2022). Regardless of the extender used, no significant differences were observed in sperm viability, acrosome integrity, or abnormalities, indicating that all extenders were effective in maintaining sperm quality during freezing and thawing.

All extenders contain stable buffering systems and energy sources that maintain osmotic balance and support sperm metabolism, respectively. Acrosome integrity and sperm viability are essential physiological functions of sperm that maintain cellular homeostasis and facilitate the transfer of genetic material to oocytes (Safa *et al*., 2025). The Indonesian National Standard has set a minimum post-thaw progressive motility threshold of 40% (BSN, 2024), which was met by all extenders examined in this study. Therefore, they were capable of preserving sperm quality within the acceptable range.

All sperm kinematic parameters were within the normal range for Belgian Blue bull semen. Sperm diluted in all extenders showed satisfactory fertilizing capability, as evinced by sperm reaching and penetrating the oocyte's zona pellucida. Oliveira *et al*. (2013) noted that the key sperm motion variables VAP, VCL, VSL, ALH, and BCF are reliable indicators of sperm fertility and are strongly associated with conception rates. Among these variables, VCL and VSL are positively correlated with fertilization ability, whereas VAP is strongly correlated with pregnancy rates.

This study has several limitations. Semen characteristics are affected by environmental and management factors, such as nutrition, temperature, and collection frequency (Kudratullah *et al*., 2024). This research focused on semen quality, which is typically determined by assessing sperm motility, viability, and abnormality. Future studies should incorporate biochemical or molecular analyses to better understand the mechanisms of cryotolerance and fertility potential in Belgian Blue bulls.

CONCLUSION

Results of this study reveal that semen characteristics among Belgian Blue bulls vary individually, reflecting variability in reproductive performance. Although TEY, AndroMed®, BoviFree®, and Steridyl® extenders effectively preserved sperm during semen freezing and thawing, sperm treated with TEY showed the highest post-thaw motility, indicating superior cryoprotective performance in Belgian Blue semen. These findings suggest that selecting appropriate extenders, particularly TEY, may enhance the efficiency of semen cryopreservation protocols and improve the success of artificial insemination programs in Belgian Blue cattle. Furthermore, accounting for individual bull variability in semen quality is essential for optimizing breeding strategies and maximizing reproductive outcomes in this breed.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used Grammarly for language improvement, grammar correction, and structural suggestions to enhance clarity and readability. Additionally, Turnitin was used to assess the manuscript's originality, and GPTZero was used to verify its human authorship. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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