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Effects of Adding Glutathione to AndroMed Diluent on Intact Plasma and Acrosome Membranes and Progressive Motility of Cattle Spermatozoa During Freezing Processes

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ABSTRACT

Adding endogenous antioxidants to the diluent is significantly associated with semen quality during the freezing process. This study aimed to investigate the effects of adding glutathione to AndroMed diluent on the preservation of crucial sperm attributes, namely, intact plasma membrane (IPM), intact acrosome membrane (IAM), and progressive motility of Bali cattle spermatozoa. A completely randomized design was used, and spermatozoa samples were obtained from a Bali cattle and divided into two diluent treatment groups (36 diluent samples in each group with six replications), namely pure AndroMed as the control and a group with the addition of glutathione (1 mmol) to AndroMed. Each treatment was replicated six times and evaluated at three freezing stages, including post-dilution, post-equilibration, and post-thawing, for crucial sperm properties. The results indicated that fresh Bali cattle spermatozoa had progressive motility, IAM, and IPM of 75%, 89%, and 88%, respectively. During the freezing process, there was a significant decrease in semen quality, including progressive motility, IAM, and IPM of spermatozoa after dilution to post-equilibration and post-equilibration to post-thawing in both treatment groups. Meanwhile, the addition of 1 mmol of glutathione to AndroMed diluent had a significant difference in increasing progressive motility, IAM, and IPM of Bali cattle spermatozoa at each stage of semen freezing, including post-dilution, post-equilibration, and post thawing when compared with controls. Based on the results, it can be concluded that adding 1 mmol of glutathione to the AndroMed diluent enhanced the quality and integrity of Bali cattle semen, including progressive motility, IAM, and IPM during the freezing process.

Keywords: Bali cattle, Freezing Process, Glutathione, Progressive Motility

INTRODUCTION

The success of an artificial insemination program using frozen semen depends on the quantity and quality of semen ejaculated by a male and the freezing process. This process begins with semen collection, evaluation, dilution, filling and sealing, equilibration, aerating over liquid nitrogen vapor, immersion in liquid nitrogen, and thawing (Bebas et al., 2018; Bebas and Agustina, 2022).

During the semen freezing process, spermatozoa are subjected to extremely low temperatures of up to -196°C, which affects the integrity of the cell membranes. At these low temperatures, the interior of cells undergoes physical and chemical changes, including increased intracellular electrolyte concentrations and the formation of ice crystals, causing cold shock (Anwar et al., 2015; Liu et al., 2021; Carriço et al., 2023). Cold shock damages the plasma and acrosome membranes (Ax et al., 2000; Bebas et al., 2018). Another primary issue faced during semen freezing is the exposure of spermatozoa to free radicals (Zhang et al., 2021; Bebas and Agustina, 2022). During this process, spermatozoa undergo peroxidation that generates free radicals such as hydroxyl (-OH) and singlet oxygen or $^1[O]_2$ (Bansal and Bilaspuri, 2011; Park and Yu, 2017). These radicals are highly reactive and potentially induce lipid peroxidation in the plasma and acrosome membranes (Douard et al., 2003; Bebas and Agustina, 2022). Lipid oxidation in the plasma membrane produces malondialdehyde (MDA) as a marker for the presence of toxic free radicals, which reduces motility and causes DNA damage (Dutta et al., 2019).

The plasma membrane regulates the influx and efflux of all electrolytes and substrates the spermatozoa needs (Delgado-Bermúdez et al., 2022). The physiological integrity is closely associated with protecting and maintaining spermatozoa motility during storage, within the female reproductive tract, capacitation, and fertilization. This is particularly significant due to the direct or indirect interactions and adhesion between the plasma membrane and cumulus oophorus (Arvioges et al., 2021; Syafi'i and Rosadi, 2022).

The acrosome membrane is a structure that covers two-thirds of the anterior head. It contains acrosin, hyaluronidase, and corona-penetrating enzyme, which function to lyse the zona pellucida as a pathway for spermatozoa

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to enter the cytoplasm of the ovum during fertilization (Nofa et al., 2017). This membrane is vital in assessing spermatozoa quality, as it is crucial for successful fertilization. Damage to the membrane leads to the leakage of enzymes and a decrease in fertilization capacity (Cahya et al., 2017; Arvioges et al., 2021).

AndroMed is one of the commercial semen diluents devoid of egg yolk, which is prone to microbial contamination. It is user-friendly, and the composition includes sugar, tris hydroxy-aminomethane, and glycerol as an energy source, a buffer, and cryoprotectant, respectively, alongside antibiotics to prevent bacterial growth (Juniandri and Isnaini, 2014). AndroMed also contains soybean extract rich in lecithin, minerals including sodium, magnesium, calcium, chloride, phosphorus, potassium, and manganese, as well as carbohydrates, proteins, citric acid, glycerol lecithin, fats, and glyceryl phosphoryl choline (Lestari et al., 2014; Aji et al., 2019).

Glutathione is a primary antioxidant that prevents the formation of new free radicals (Ansari et al., 2021). It converts existing free radicals into less membrane-impactful molecules. Free radicals are atoms or molecules with one or more unpaired electrons and are highly unstable (Zulaikhah, 2017). To obtain a pair, free radicals react with other atoms or molecules, such as unsaturated fatty acids, proteins, nucleic acids, or lipopolysaccharides, resulting in the creation of abnormal compounds (Pizzorno, 2014; Ansari et al., 2021). The addition of glutathione to the spermatozoa diluent medium reduces or prevents the formation of free radicals that could damage plasma and acrosome membranes as well as motility. Consequently, the fertility of frozen semen increases, ultimately leading to higher pregnancy rates. The recommended concentration of glutathione suitable as a diluent for cattle semen freezing is approximately 1 mmol/L (Syarifuddin et al., 2012), and for dairy goats is 2 mmol/L (Zou et al., 2021). Based on the information provided, this study aimed to determine the impact of adding glutathione to AndroMed diluent on the intact plasma membrane, intact acrosome membrane, and progressive motility of Bali cattle spermatozoa in the post-dilution, post-equilibration, and post-thawing processes. This research reveals new information regarding the role of glutathione as an antioxidant added to Andromed semen diluent on the quality of Bali cattle semen during the freezing process.

MATERIALS AND METHODS

Ethical approval

All procedures have been reviewed by the Experimental Animal Ethics Committee, Faculty of Veterinary Medicine, Udayana University, and have received approval No. B/252/UN14.2.9/PT.01.04/2023.

Materials and Equipment

The materials used included 4-year-old Bali cattle semen, AndroMed (REF:13503/0200 Minitub, Hauptstrasse-Germany), Glutathione (248-170-7 Merck, Darmstadt-Germany), NaCl crystals (1.06404.0500 Merck, Darmstadt-Germany), Sodium citrate (1.06448.0500 Merck, Darmstadt-Germany), Fructose (1.05323 Merck, Darmstadt-Germany), Alcohol 70%, distilled water, liquid nitrogen, and Formalin solution 37% (Merck, Darmstadt-Germany).

The equipment utilized included binocular microscope, spectrophotometer, micropipette, Pasteur pipette, 10 ml volumetric pipette, glass slides, cover slips, pH meter, measuring cylinder, Erlenmeyer flask, straws, straw rack, water bath, 1 cc and 10 cc syringes, livestock crush, artificial cattle vagina, refrigerator, Styrofoam box, filling and sealing machine, straw printing machine, and liquid nitrogen container.

Semen shelter for Bali cattle

The experimental animal utilized in this research was a 4-year-old male Bali cattle, in healthy condition according to veterinary examination. The research was conducted from May to September 2023 at the Artificial Insemination and Livestock Breeding Center, Baturiti District, Tabanan Regency, Bali Province, Indonesia. Before semen collection, flushing of the preputium was performed to ensure cleanliness, followed by drying with a clean cloth. The teaser cattle were introduced into the mating pen, then the male was brought closer, allowed to mount the teaser cattle, and subsequently dismounted (Mappanganro, 2020). This process was repeated 2-3 times to enhance the libido of the male. Once an erection occurred, the penis was directed towards the artificial vagina by holding the preputial region, followed by ejaculation. The collected ejaculate of approximately 5-6 ml was promptly transported to the laboratory and placed for 3-5 minutes in a water bath at 37^{o}C for immediate semen quality evaluation at the Laboratory of Artificial Insemination and Livestock Breeding.

Semen evaluation

The collected semen from a single male was subjected to both macroscopic and microscopic examinations. The research design used a completely randomized design, where 72 spermatozoa samples were divided into two treatment groups, with three stages of the freezing process and each with six replications. The macroscopic examination entailed evaluations of volume, pH, consistency/viscosity, color, and odor. Meanwhile, the microscopic examination using Microscope Binoculer Olympic CX-23 (Olympus Corporation, Japan) with magnification 400x included analyses of

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mass motility, individual motility, spermatozoa concentration, abnormality, IPM, and IAM (Cocchia et al., 2011; Susilawati, 2011).

AndroMed diluent preparation

AndroMed in powdered form (grams) was placed into a measuring glass (50 ml) containing distilled water with a ratio of 1 (gram) to 4 (ml), then homogenized. Afterward, the sample was placed in a water bath for 3-5 minutes at 37ºC, and the diluent was prepared for use (Susilawati, 2011). For the test group, glutathione was added to AndroMed diluent at a concentration of 1 mmol, according to previous studies (Syarifuddin et al., 2012; Maleki et al., 2023).

Cement dilution

The collected semen and the AndroMed diluent were placed for 3-5 minutes in a water bath at a temperature of 37°C. This step was taken to equilibrate the temperature of the diluent with that of the semen. Furthermore, semen that underwent evaluation was diluted using the diluent prepared according to the formula for calculating the diluent quantity (Wishart, 2009; Syarifuddin et al., 2012).

Straw printing

For liquid semen that fulfilled the quality standards before freezing, the next step entailed labeling the packaging using a straw printing machine (Minitub, Hauptstrasse-Germany) (Syarifuddin et al., 2012; Maleki et al., 2023).

Filling and sealing

Liquid semen that fulfilled the standards was filled into mini straws with a volume of 0.25 ml, containing a concentration of 25 million spermatozoa (Syarifuddin et al., 2012; Setiono et al., 2015). Subsequently, the packaging was sealed using a filling and sealing machine (Minitub, Hauptstrasse-Germany).

Equilibration

The semen that underwent filling and sealing was then stored in a refrigerator at a temperature of 4°C for 4 hours (Murphy et al., 2018; Swarna et al., 2023).

Aerating in liquid nitrogen vapor

The pre-freezing process was conducted by rapidly lowering the semen temperature from 4°C to -140°C to prevent cold shock (sudden temperature change affecting sperm cells). It was accomplished by placing straws, arranged on a straw rack, into the liquid nitrogen vapor approximately 4 cm above the surface for 9 minutes (Syarifuddin et al., 2012; Baharum et al., 2017).

Freezing

The freezing process was carried out by immersing the semen product into liquid nitrogen until fully submerged, resulting in the temperature dropping to -196°C (Ansari et al., 2021).

Post-thawing motility evaluation

In the Post-thawing motility (PTM) process, frozen semen was subjected to quality testing to determine its suitability for distribution according to the Indonesian National Standards, which required a minimum of 40% progressive motility and a minimum concentration of 25 million cells per dose. Frozen semen that did not fulfill these standards was subsequently discarded. In this research, the assessment of semen quality was achieved by observing progressive motility, IPM, and IAM. These observations were conducted on fresh semen post-dilution with respective treatments, post-equilibration, and post-thawing (Zhang et al., 2021).

Progressive motility examination

Diluted semen of 0.05 ml was gently dropped onto a warm glass slide (37°C), covered with a glass cover, and observed under a light Microscope Binoculer Olympic CX-23 (Olympus Corporation, Japan) at 400x magnification. The counting of spermatozoa exhibiting progressive motility was performed and expressed as a percentage. This observation was carried out across five fields of view (Wishart, 2009; Manehat et al., 2021).

Intact plasma membrane examination

The percentage of spermatozoa IPM was assessed using the hypoosmotic swelling (HOS) test method (Nur et al., 2012). The composition of the hypoosmotic solution consisted of 0.9g of fructose and 0.49g of sodium citrate dissolved in distilled water to reach a volume of 100 ml (100 mOsm/kg). About 20 ml of the solution was combined with 0.2 ml of semen, mixed to homogeneity, and then incubated at 37°C for 45 minutes. Thin smears were prepared on glass slides,

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followed by evaluation under a light microscope Binoculer Olympic CX-23 (Olympus Corporation, Japan) at 400x magnification on a minimum of 200 spermatozoa. Spermatozoa with IPM were characterized by coiled or swelling tails, while damaged ones exhibited straight tails.

Intact acrosome membrane examination

About 0.9 grams of NaCl was dissolved in distilled water to make 100 ml, then 1 ml of formalin was added to 99 ml of the physiological NaCl solution and shaken until homogeneous. A part of semen was mixed with three parts of the NaCl and formalin mixture. The mixture was left for approximately 3 minutes, and thin smears were created on glass slides. Spermatozoa were examined under a light microscope at 400x magnification. The assessment was conducted by counting the proportion with IAM within 100 spermatozoa. Samples with IAM were marked by the presence of a dark acrosome membrane (Cocchia et al., 2011; Zhang et al., 2021).

Data analysis

The obtained data were analyzed using analysis of variance (ANOVA) through SPSS version 25 for Windows. In cases of significant differences among treatments, further analysis was conducted using the Duncan test. P value < 0.05 is declared statistically significant.

RESULTS

The macroscopic evaluation of fresh Bali cattle semen yielded normal characteristic aroma, moderate-normal consistency, creamy color, and pH of 6.5. Meanwhile, the microscopic evaluation showed mass motility (+++), spermatozoa concentration of $1358x10^6$ ml^{-1}, 75% progressive motility, 86% viability, 5% abnormality, IPM at 86%, and IAM at 89%. The comprehensive results of the semen evaluation are presented in Table 1. The addition of glutathione with a concentration of 1 mmol to the AndroMed diluent led to an improvement in semen quality during the freezing process stages, including dilution, post-equilibration, and post-thawing. Statistical analysis indicated that the treatment and freezing stages significantly ($p < 0.05$) affected the quality of Bali cattle semen, compared to control (Table 2). The effect of adding glutathione to the AndroMed diluent on the semen quality during the freezing process stages is shown in Figures 1, 2, and 3. Microphotograph of sperm motility and integrity are shown in Figures 4, 5, and 6.

Table 1 shows that fresh Bali cattle spermatozoa have progressive motility, IAM, and IPM of 75%, 89%, and 88% respectively. Meanwhile, Table 2 shows that the stages of the freezing process had a significant effect ($p < 0.05$) on reducing progressive motility, IAM, and IPM of spermatozoa after dilution compared to post-equilibration and post-equilibration, compared to post-thawing in all treatment groups. Next, the obtained showed that the addition of 1 mmol gluthation to Andromed diluent had a significant difference ($p < 0.05$) in increasing progressive motility, IAM, and IPM of Bali cattle spermatozoa at the post-dilution, post-equilibration, and post thawing compared to control.

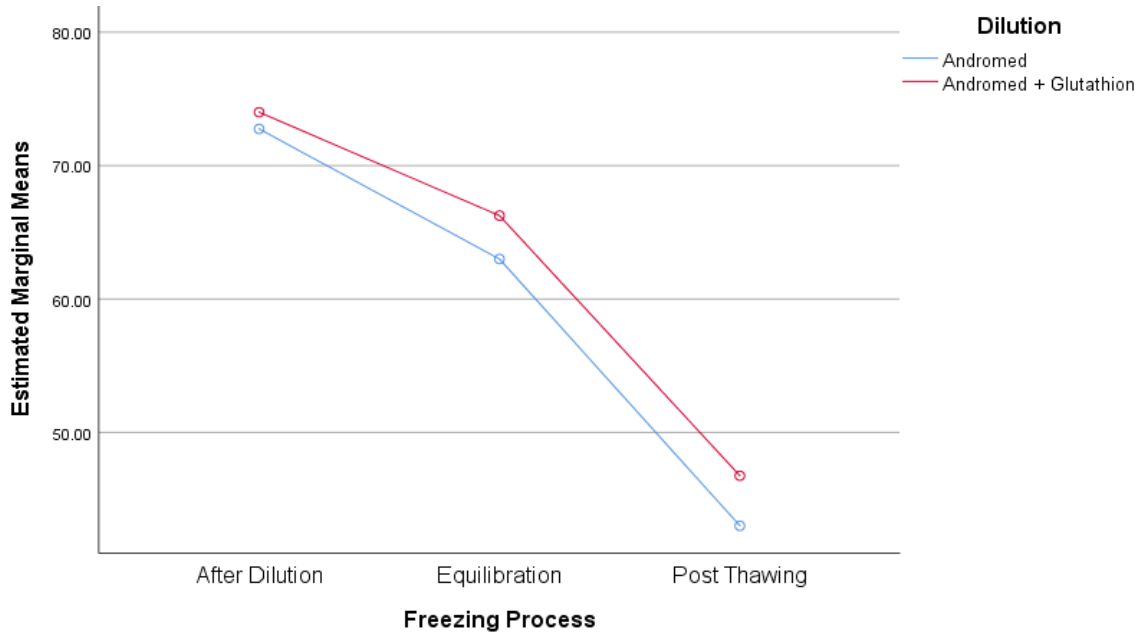

Figure 1. Motility of 4 years old Bali cattle spermatozoa due to the effect of adding 1 mmol glutathione to AndroMed diluent during the semen freezing process

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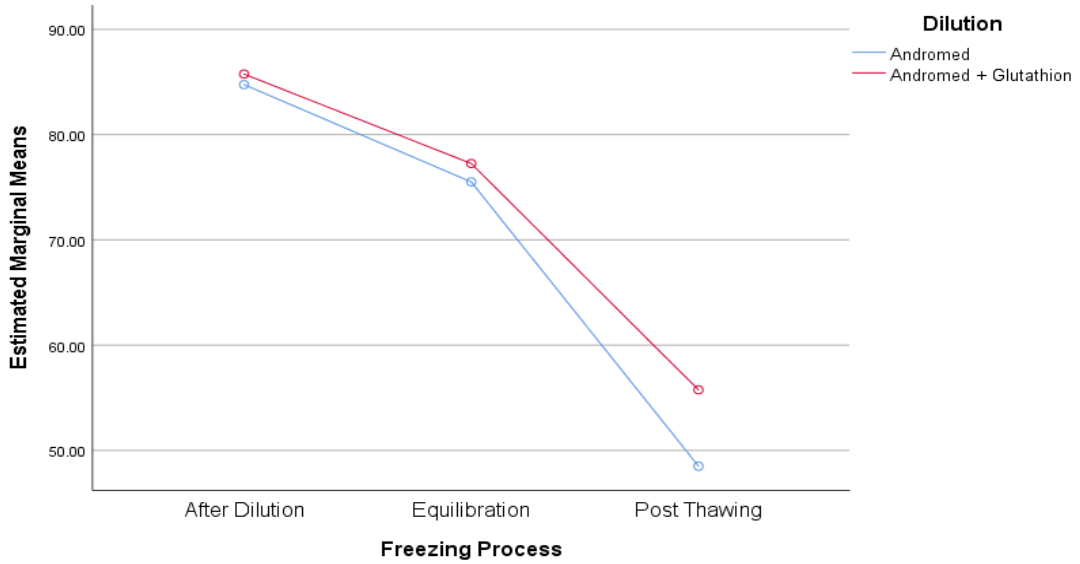

Figure 2. Intact acrosome membrane of 4 years old Bali cattle spermatozoa due to the effect of adding 1 mmol glutathione to AndroMed diluent during the semen freezing process

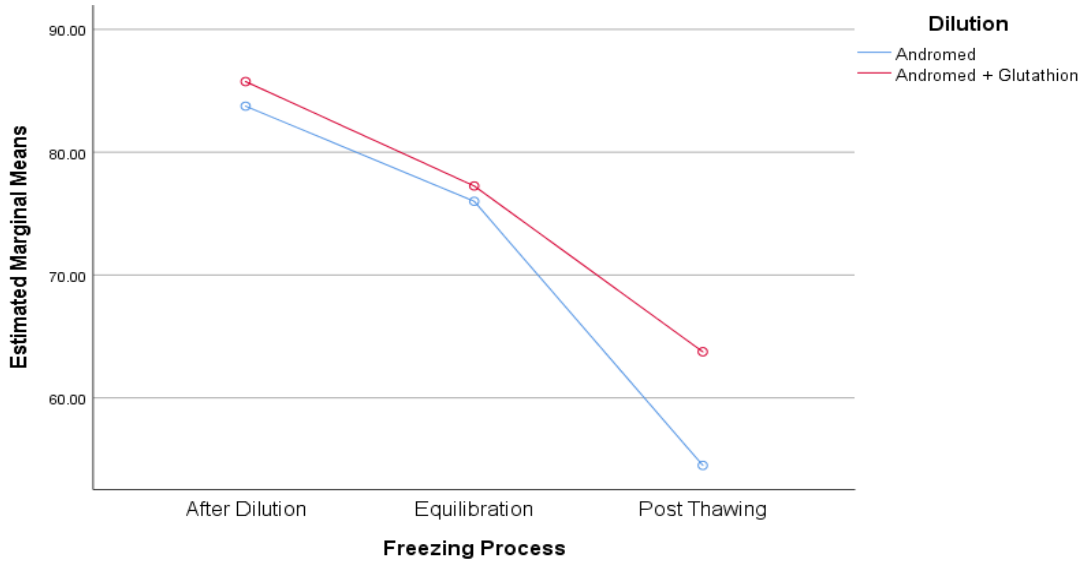

Figure 3. Intact plasma membrane of 4 years old Bali cattle spermatozoa due to the effect of adding 1 mmol glutathione to AndroMed diluent during the semen freezing process

Figure 4. Micropothograph of 4 years old Bali cattle spermatozoa with normal morphology (400x).

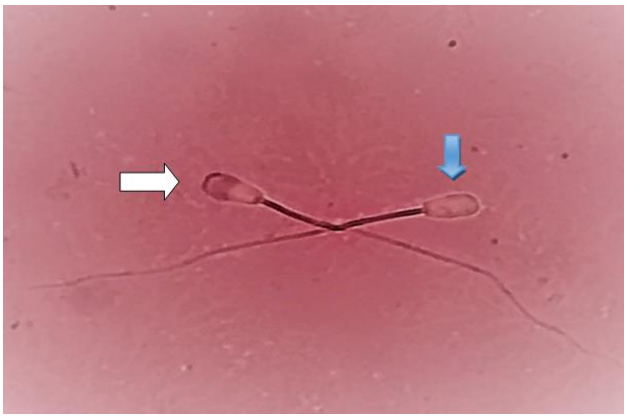

Figure 5. Microphotograph of 4 years old Bali cattle spermatozoa with intact acrosome membrane (white arrow) and detached acrosome membrane (blue arrow, 400x)

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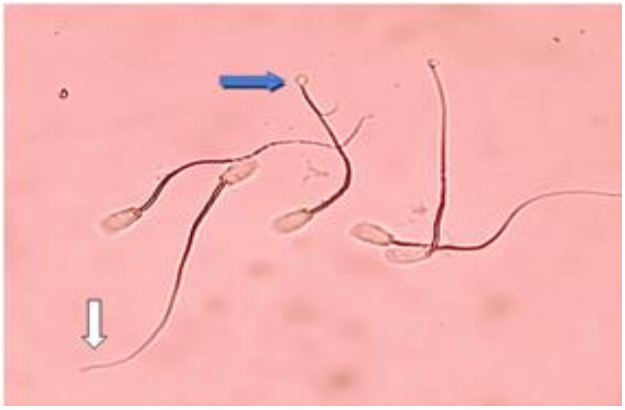

Figure 6. Microphotograph of 4-year-old Bali cattle spermatozoa with intact plasma membrane and curled tail (blue arrow) and detached plasma membrane with straight tail (white arrow, 400x)

Table 1. Macroscopic and microscopic fresh semen quality of Bali cattle aged 4 years

Parameter		Value
Macroscopic	Volume (ml)	5.8
	Odor	Normal
	Consistency	Moderate-thick
	Color	Cream
	pH	6.5
Microscopic	Mass Motility	+++
	Spermatozoa Concentration ($x10^6$)	1358
	Progressive Motility (%)	75
	Viability (%)	86
	Abnormality (%)	5
	Intact Acrosome Membrane (%)	89
	Intact Plasma Membrane (%)	88

+++: Mass motility was very good

Table 2. Effect of freezing stages on semen quality of Bali Cattle aged 4 years diluted using AndroMed with the addition of 1 mmol Glutathione

Parameter	Freezing stages	Diluted	
		AndroMed	AndroMed + 1 mmol Glutathione
Progressive motility (%)	Post-Dilution	72.75 ± 1.25^{Aa}	74.38 ± 1.15^{Ba}
	Post-Equilibration	63.00 ± 1.82^{Ab}	66.25 ± 0.95^{Bb}
	Past-Thawing	43.00 ± 0.81^{Ac}	46.75 ± 0.95^{Bc}
Intact acrosome membrane (%)	Post-Dilution	84.75 ± 0.95^{Aa}	85.75 ± 1.71^{Ba}
	Post-Equilibration	75.50 ± 1.29^{Ab}	77.25 ± 1.70^{Bb}
	Past-Thawing	48.50 ± 1.29^{Ac}	55.75 ± 2.06^{Bc}
Intact plasma membrane (%)	Post-Dilution	83.75 ± 0.95^{Aa}	85.25 ± 0.95^{Ba}
	Post-Equilibration	76.00 ± 1.41^{Ab}	77.25 ± 1.50^{Bb}
	Past-Thawing	54.50 ± 1.29^{Ac}	63.75 ± 1.89^{Bc}

^{abc}Superscript letters that differ towards the column show a significant difference ($p < 0.05$); ^{ABC}Different superscripts letters towards the row indicate significant differences ($p < 0.05$)

DISCUSSION

The results from this research (Table 1) were consistent with Bebas et al. (2022), where macroscopic evaluation such as volume, odor, consistency, color, and pH was reported as 5.98±1.35 (Bebas and Agustina, 2022). Meanwhile, microscopic evaluation, including mass motility, concentration, progressive motility, abnormality, and IPM was noted as +++, 1104 ± 202.21 x 10^6 ml^{-1} of ejaculate, 69.58±0.30%, 4.66±1.58%, and 83.22±1.64%, respectively. Hafez and Hafez (2000) stated that normal semen appeared whitish-gray to pale cream in color, with a thick consistency. The consistency correlates with the color; for instance, creamy-colored semen tends to have a thick or viscous consistency, while clear or light-colored semen is usually less viscous (Islam et al., 2018). The distinct aroma of cattle semen indicates normalcy and the absence of contamination. This aligned with Ilaria (2011), which categorized semen odor as distinctive. According to Hafez and Hafez (Hafez and Hafez, 2000), the concentration of cattle spermatozoa ranges between 800-2000 million/ml. The observed mass motility was very good (+++), characterized by large, numerous, dark, thick, active, and fast-moving waves. In general, the cattle semen fell within the normal concentration with good quality and integrity, in line with reports by Garner and Hafez (2000) and Prastowo et al. (2018).

During the semen freezing process, including dilution and equilibration, spermatozoa encounter extremely low and extreme temperatures, reaching -196°C. Freezing significantly affects the cell membrane of spermatozoa, with subzero temperatures below the freezing point causing physical and chemical changes, including the formation of ice crystals and an increase in intracellular electrolyte concentration, resulting in cold shock (Pratiwi et al., 2014; Çelik et al., 2020). According to previous research, cold shock culminates in damage to both plasma and acrosome membranes (Liu et al., 2021; Carriço et al., 2023). The main challenge encountered during semen freezing was not only cold shock but also the exposure of spermatozoa to free radical attacks such as hydroxyl and singlet oxygen (Park and Yu, 2017; Angrimani et al., 2018). These highly reactive radicals can induce lipid peroxidation on plasma and acrosome membranes (Bansal and

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Bilaspuri, 2011; Syafitri et al., 2022). The spermatozoa plasma membrane consists of double lipids containing unsaturated fatty acids which are very susceptible to peroxidation (Douard et al., 2003). Free radicals work to oxidize lipids in the membrane so that the unsaturated fatty acid chains are broken and produce MDA, which is toxic to spermatozoa cells (Zulaikhah, 2017; Zhang et al., 2021). The presence of MDA is an indicator of the existence of free radicals (Dutta et al., 2019) that damage the plasma membrane, resulting in a decrease in the quality and integrity of spermatozoa, including concentration, motility, and DNA damage (Prihantoko et al., 2022).

Research related to antioxidants as cryoprotectants has reported success in reducing oxidative damage due to reactive oxygen species (Zulaikhah, 2017; Liu et al., 2021). Glutathione is a significant antioxidant that neutralizes free radicals for cryopreserved in dogs (Angrimani et al., 2018), Indian red jungle fowl (Ansari et al., 2021), Mithun cattle (Perumal et al., 2021), goat (Zou et al., 2021) and sheep (Syafitri et al., 2022). It is a naturally occurring substance present in the body since birth and is found both within and outside cells (Zou et al., 2021). Blood glutathione levels range from 5 to 8 mM/L, with the highest concentration being present in the liver, which serves as the most essential organ for detoxification in the body. This antioxidant is also present in the spleen, kidneys, lungs, heart, brain, and stomach (Zulaikhah, 2017).

The glutathione antioxidant system serves as a primary endogenous protection mechanism, actively participating in the neutralization of reactive oxygen species (ROS) and maintaining the reduced (active) forms of vitamins C and E (Biswas et al., 2020). Glutathione is also called the "master antioxidant" as it could lead to the activity of other antioxidants (Chakravorty et al., 2020). For instance, vitamins C and E capture free radicals, followed by the transfer to glutathione, which then cycles to capture more ROS (Zeitoun and Al-Damegh, 2014). This antioxidant neutralizes and eliminates free radicals through urine. Its efficacy in protecting cells surpasses other antioxidants, including vitamins C and E. Furthermore, glutathione protects DNA and RNA chains from degradation and shields the nucleus from free radicals. The glutathione binds and expels unwanted substances through urine and bile (Solihati et al., 2018). The presence of glutathione protects cells from toxic ROS effects and acts as a scavenger for hydroxyl (-OH) and superoxide (O_2^-) radicals. This antioxidant also plays roles in maintaining cell survival, DNA replication, protein thiolation, enzyme catalysis, membrane transport, receptor action, intermediary metabolism, and cell maturation (Zhang et al., 2005). According to a previous study, glutathione captures peroxides that could harm cells. Due to the mitochondrial respiration process, all aerobic organisms naturally face oxidative stress. Compounds, including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), induce the production of oxygen radicals, which are toxic and capable of causing lipid peroxidation and cellular injury (Zeitoun and Al-Damegh, 2014; Bollwein and Bittner, 2018). Based on the results, adding glutathione at a concentration of 1 mmol to the AndroMed diluent significantly improved semen quality, including spermatozoa motility, IPM, and IAM, during the freezing process stages.

CONCLUSION

The addition of glutathione with a concentration of 1 mmol to the AndroMed diluent significantly improved the frozen semen quality characterized by progressive motility, intact acrosome membrane, and intact plasma membrane during the freezing process stages, including post-dilution, post-equilibration, and post-thawing. Furthermore, research needs to be carried out on fertility tests on semen quality through artificial insemination technology.

DECLARATIONS

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Availability of data and materials

The data of the current study are available.

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Authors' contributions

Wayan Bebas is the Conception and design of the study. Wayan Bebas and I Wayan Gorda conducted an Acquisition of data. Wayan Bebas and I Made Merdana Analysis and interpretation of data, and wrote the manuscript.

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Kadek Karang Agustina and I Wayan Gorda give the Critical review and revision. All authors checked and confirmed the data analysis and the final version of manuscript.

Competing interests

We declare there is no any conflict of interest in the publication of this article.

Ethical consideration

Ethical issues, such as data fabrication, double publication and submission, redundancy, plagiarism, consent to publish, and misconduct, have been checked by all the authors before publication in this journal.

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