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Original article

Addition of basil leaf (*Ocimum basicilum* L.) extract to AndroMed® extender improved the quality of spermatozoa of frozen thawed Simmental bull semen

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ABSTRACT

This study aims to determine the best dose of basil leaf extract to add to AndroMed® semen extender to improve spermatozoa quality after freezing and thawing. This study used healthy, normal four-year-old Simmental bulls with good libido. The sample used was fresh semen from Simmental bull collected using an artificial vagina. This study used a completely randomized design (CRD) with three treatments and six replicates. Ejaculate that met the motility qualification of more than 70%, was divided into three groups, each extended in AndroMed® without the addition of basil leaf extract (T0), with the addition of 0.1% basil leaf extract (T1) and with the addition of 0.3% basil leaf extract (T2). Straw containing semen with 30 million spermatozoa/straw was processed using an IceCube automatic freezer (Minitube, Germany). The results showed that spermatozoa motility, viability, and spermatozoa plasma membrane integrity in the T2 group were higher ($p < 0.05$) than those in the T0 and T1 groups, while spermatozoa morphological abnormalities in the T2 group were lower ($p < 0.05$) than those in the T0 and T1 groups. There were no significant differences ($p > 0.05$) in these parameters between groups T0 and T1. It could be concluded that the addition of 0.3% basil leaf extract to the AndroMed® extender could improve the quality of the semen of Simmental bulls after thawing.

Keywords: antioxidant, morphological abnormalities, motility, plasma membrane integrity, viability

INTRODUCTION

Frozen semen is diluted in an extender with the aim of ensuring the survival of spermatozoa. Extenders are chemical substances designed to preserve, maintain and protect spermatozoa from potential damage that may occur during processing, storage and distribution in artificial insemination (AI). Its main function was to provide an energy source for spermatozoa,

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thereby ensuring their survival during storage or freezing. An effective extender must provide nutrition, maintain optimal pH, has proper osmotic pressure, and protect spermatozoa from cold shock (Bustani *et al*., 2021). Some commercial extenders had been used to freeze bull semen in some artificial insemination centers around the world. One example of a commercial extender is AndroMed®, which is tris-based and specially formulated for frozen bovine semen. The composition includes tris-(hydroxymethyl)-aminomethane as a buffer, glucose as an energy source, glycerol as a cryoprotectant, and antibiotics to inhibit bacterial growth (Kamal *et al*., 2022). AndroMed® does not contain egg yolk, reducing contamination risks from microorganisms and enhancing its handling and storage capabilities. As a yolk-free medium, AndroMed® was associated with high fertility rates for both frozen and liquid semen (Bustani *et al*., 2021).

Despite its benefits, one significant challenge during semen freezing process was the reduction in motility and viability of spermatozoa, which could be attributed to damage caused by free radicals (Vigolo *et al*., 2022). A study on buck frozen semen diluted with Tris amino methane-based extender without the addition of egg yolk showed a higher membrane damage after thawing (Kamal *et al*., 2022). Another study reported that adding antioxidants to extenders could help minimize membrane damage caused by lipid peroxidation during the cooling process (Qamar *et al*., 2023). Additionally, mammalian spermatozoa typically lack significant cytoplasmic components to neutralize damage from reactive oxygen species (ROS) and lipid peroxidation (Villaverde *et al*., 2019).

Among potential antioxidants, flavonoids and arginine derived from basil (*Ocimum basilicum* L.) were quite promising (Romano *et al*., 2022). Flavonoids worked as antioxidants that protected DNA and counteracted free radicals (Heim *et al*., 2002). Arginine had antioxidant properties that were able to maintain the integrity of the spermatozoa plasma membrane and reduce MDA levels as an indicator of lipid peroxidation, therefore enhanced the motility and viability of spermatozoa (Susilowati *et al*., 2019). As a common semi-essential amino acid, arginine scavenged hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\bullet-}$), while also diminishing the activity of free radicals that generated nitric oxide (NO). Nitric oxide played a protective role by safeguarding spermatozoa from membrane damage due to lipid peroxidation (Kaltsas, 2023). Therefore, this study aims to determine the effect of adding several concentrations of Basil (*Ocimum basilicum* L.) leaf extract to the AndroMed® extender on motility and viability of frozen-thawed Simmental bull spermatozoa.

MATERIALS AND METHODS

This research was conducted in the Laboratory at the Teaching Farm of the Faculty of Veterinary Medicine, Universitas Airlangga, located in Tanjung village, Kedamean district, Gresik regency, East Java, Indonesia. This study used a completely randomized design with three treatments and six replicates.

Ethanol extraction of basil leaf

Fresh and clean basil leaf were selected, washed and air-dried for approximately 14 days. After drying, simplicia was sorted again to ensure that everything is clean, and processed into powder form. The basil leaf powder was then soaked in 96% ethanol solution. The maceration process was carried out over three consecutive 24-hour periods, with daily stirring. On the third day, the mixture was filtered using filter paper assisted by a vacuum pump. The resulting macerate was then evaporated using an evaporator set at 50 rpm and a temperature of 45°C until a thick ethanol extract was obtained. This extract was subsequently freeze-dried and stored at -20°C, yielding a stable freeze-dried basil leaf extract (A'yuni *et al*., 2023).

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Preparation of AndroMed® extender

AndroMed® was mixed with distilled water in a ratio of 1:4. The mixture was stirred until homogeneous and then placed in a water bath at 38°C, ready to be used as an extender for further semen processing (Pieper *et al*., 2023).

Collection and treatment of semen

Fresh semen from Simmental bulls was collected using an artificial vagina. Ejaculates that met the motility qualification of over 70% were divided into three groups based on the addition of basil leaf extract i.e. group T0 without basil leaf extract, group T1 with 0.1% basil leaf extract, and group T2 with 0.3% basil leaf extract. Prior to treatment, semen was evaluated through macroscopic and microscopic examinations. The macroscopic assessments included evaluations of volume, color, odor, consistency, and pH, while microscopic examinations focused on mass movement, progressive individual movement, viability, and concentration. Semen quality was classified as acceptable if spermatozoa exhibited more than 70% progressive movement (Susilowati *et al*., 2020).

Prefreezing and freezing of semen

Semen freezing was conducted using the IceCube automatic freezer (Minitube, Germany). The device was programmed for a temperature reduction rate of 15°C/minute, 10°C/minute, and 5°C/minute. Once programmed, the straw containing the semen (30 million spermatozoa/straw) was inserted into the device and frozen from 4°C to -120°C, and transferred to a container with liquid nitrogen (Vigolo *et al*., 2023). After frozen storage for 24 hours, the quality of the thawed semen was assessed. The semen straw was thawed in a water bath at approximately 37°C for 30 seconds and then the straw was cut at both ends to release the semen onto a glass slide for examination (Susilowati *et al*., 2021b).

Spermatozoa motility examination

Spermatozoa motility was evaluated by placing a semen sample on a glass slide and covered with a cover slip. A microscope at a magnification of 100x was utilized to assess mass movement, while 400x magnification was used for evaluating individual motility (Susilowati *et al*., 2021b; Ratnawati *et al*., 2023). Individual motility was expressed as a percentage ranging from 0 to 100% in five fields of view, then averaged.

Spermatozoa viability examination

The viability of spermatozoa was assessed using eosin-negrosin staining. The semen was homogeneously mixed with eosin-negrosin stain (2% Eosin Y solution (Bioanalytic GmbH) and 10% Nigrosin (Bioanalytic GmbH)) (Kanna and Shetty, 2023) on a glass slide and evenly spread. Spermatozoa that appeared purplish red were classified as dead, while those that were white were considered live (Susilowati *et al*., 2021b).

Spermatozoa plasma membrane integrity examination

The integrity of the spermatozoa plasma membrane was evaluated using the Hypo-osmotic Swelling Test (HOS Test). Plasma membrane integrity was determined by observing whether the spermatozoa tails were bent, circular or bulging, assessed under a microscope at 400× magnification (Ramu and Jeyendran, 2013; Susilowati *et al*., 2021b).

Spermatozoa morphological abnormality examination

Spermatozoa abnormalities were analyzed through smear preparations stained with eosin-negrosin (2% Eosin Y solution (Bioanalytic GmbH) and 10% Nigrosin (Bioanalytic GmbH)) (Kanna and Shetty, 2023). Changes in the morphology of the spermatozoa, specifically in the head and tail structures, were examined under a microscope at 400× magnification (Pahlevy *et al*., 2022; Pelzman and Sandlow, 2024).

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Data analysis

Data were analyzed using One-Way Analysis of Variance using SPSS version 20 for Windows at a 5% confidence level. If significant differences were detected, further analyses were conducted to determine the specific comparisons between groups.

RESULTS

Table 1 Simmental bull fresh semen quality

volume (mL)	9.5 ± 0.96
pH	6-7
color	creamy white
odor	specific
consistency	thick
concentration (million/mL)	1419.83 ± 230.1
individual movement (%)	80 ± 5.48
mass movement	+++

+++= very good semen quality with active dense spermatozoa forms large thick waves.

Examination of fresh semen showed good results and met the requirement for individual motility with progressive movement above 70% (Tabel 1), therefore semen was eligible for freezing. After freezing and thawing, examination showed that spermatozoa motility, viability, plasma membrane integrity of T2 group were higher ($p < 0.05$) compared to those of T0 and T1 groups, while and spermatozoa morphological abnormality of T2 was lower ($p < 0.05$) compared to those of T0 and T1 groups There was no significant different ($p > 0.05$) in these parameters between groups T0 and T1 (Table 2).

Table 2 Spermatozoa motility, viability, plasma membrane integrity and abnormality of frozen thawed Simmental bull semen (means ± SD)

	motility	viability	PMI	abnormality
T0	40.55 ± 2.27 ^{a}	55.35 ± 1.75 ^{a}	29.62 ± 3.04 ^{a}	10.44 ± 1.25 ^{b}
T1	40.83 ± 2.29 ^{a}	56.73 ± 1.61 ^{a}	30.97 ± 2.44 ^{a}	9.49 ± 0.74 ^{ab}
T2	44.17 ± 1.39 ^{b}	60.43 ± 2.01 ^{b}	34.29 ± 2.09 ^{b}	8.89 ± 0.90 ^{a}

Different superscripts in the same column indicate significant differences ($p < 0.05$); T0: semen extended in AndroMed®; T1: semen extended in AndroMed® contained 0.1% basil leaf extract; T2: semen extended in AndroMed® contained 0.3% basil leaf extract; PMI: plasma membrane integrity.

Figure 1 Examination of spermatozoa viability with eosin nigrosin staining (Nikon Eclipse E200, 400x magnification); a: spermatozoa whose heads are unstained indicate live spermatozoa; b: spermatozoa whose heads are purplish red indicate dead spermatozoa.

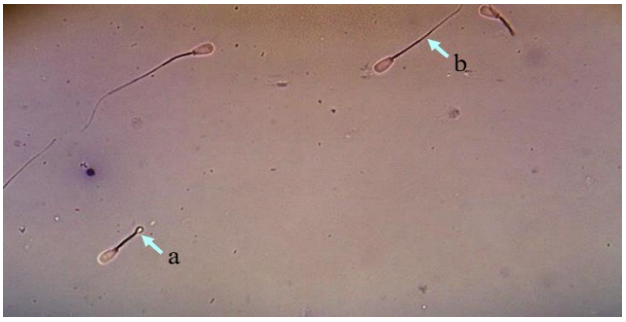

Figure 2 Examination of plasma membrane integrity of frozen thawed Simmental bull spermatozoa (Nikon Eclipse E200, 400x magnification); a: intact plasma membrane integrity is indicated by a bulging or coiled tail; b: damaged plasma membrane integrity is indicated by a straight tail.

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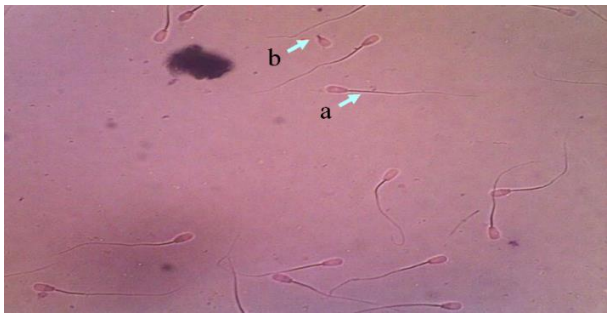

Figure 3 Abnormalities of frozen thawed Simmental bull spermatozoa (Nikon Eclipse E200, 400x magnification); a: normal spermatozoa morphology is characterized by normal head and tail formation; b: abnormalities of spermatozoa are characterized by head formation without a tail.

DISCUSSION

The parameters of fresh semen from Simmental cattle (volume, odor, pH, color, consistency, individual motility, mass movement, and concentration) in this study were in the range of previous research results (Susilowati *et al*., 2021). Overall, the fresh Simmental bull semen examined demonstrated good quality and was suitable for processing into frozen semen.

Spermatozoa motility

According to Indonesian national standards (SNI 4869-1:2021), frozen thawed bovine semen must contain spermatozoa with a minimum motility of 40%. The addition of 0.3% basil leaf extract to AndroMed® extender resulted in the highest (more than 40%) progressive spermatozoa motility. Spermatozoa motility was closely associated with the integrity of the plasma membrane (Shan *et al*., 2021). During freezing, spermatozoa membrane underwent changes that could damage its lipid composition, primarily due to lipid peroxidation, which affected membranes rich in phospholipids and unsaturated fatty acids. The damage of spermatozoa plasma membrane in the freezing-thawing process led to reduced motility (Carro *et al*., 2022).

This study found that adding basil leaf extract in appropriate dosages enhanced spermatozoa motility compared to conditions without the extract. Flavonoids in basil might play a critical role in neutralizing free radicals during storage, minimizing declines in motility. Proper antioxidant administration helped protect spermatozoa plasma membranes, which were susceptible to lipid peroxidation (Mishra *et al*., 2024). Arginine was also essential in protecting the spermatozoa membrane from lipid peroxidation by increasing NO production through NOS in the acrosome, enhancing intercellular signaling (Kaltsas *et al*., 2023). Nitric oxide acted as an antioxidant by disrupting free radical chain reactions and enhanced the synthesis of cyclic guanosine monophosphate (cGMP), positively impacting metabolic rates (Dutta *et al*., 2022). The conversion of ATP to cyclic Adenosine Monophosphate (cAMP) acted as a key second messenger that enhanced the activity of protein kinase A (PKA), which was crucial for spermatozoa movement (Buffone *et al*., 2014). In summary, arginine helps neutralize free radicals, boosts NO production, and supported the energy metabolism needed for the motility of spermatozoa, with ATP levels being closely linked to spermatozoa metabolism (Susilowati *et al*., 2019).

Spermatozoa viability

The results of this study indicate that the antioxidant components of flavonoids and arginine in the extender played a crucial role in preserving the quality of spermatozoa against damage caused by free radicals. Basil leaves contained a variety of chemical compounds, including essential oils, flavonoids, phenylpropanoids, and rosmarinic acid (Kamelnia *et al*., 2023). Among these, flavonoids acted as potent antioxidants, effectively neutralizing free radicals and thus preventing cellular damage (Chagas *et al*., 2022). Flavonoids were particularly effective in maintaining spermatozoa quality by preserving their motility. Enhanced motility not only

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increased viability but also contributed to a higher overall number of viable spermatozoa. Antioxidants played a vital role in inhibiting lipid peroxidation reactions, which could severely damage the membranes of spermatozoa during the freezing process. Flavonoids could efficiently scavenge free radicals, thereby maintaining the integrity of spermatozoa plasma membranes. Preserving the integrity of these membranes enhanced their durability and prolongs the lifespan of the spermatozoa, leading to a higher percentage of living cells (Qamar *et al*., 2023).

Furthermore, administering antioxidants at the appropriate dosage was essential to maximize protection for the plasma membranes of spermatozoa, which were rich in unsaturated fatty acids and particularly susceptible to lipid peroxidation (Kowalczyk, 2021). Oxidation involved a chemical reaction that transfers electrons from one substance to an oxidizer, generating free radicals that could instigate destructive chain reactions within cells (Martemucci *et al*., 2022). In addition to flavonoids, arginine played a significant role in disrupting these chain reactions by binding to unpaired electrons, effectively inhibiting additional oxidation reactions. In biological systems, antioxidants like arginine served as electron donors that mitigated the detrimental effects of oxidants, including free radicals, enzymes, and metal-binding proteins (Hassanpour *et al*., 2023).

Spermatozoa plasma membrane integrity

The evaluation of plasma membrane integrity in spermatozoa revealed that the addition of basil leaf extract to the AndroMed® extender significantly enhanced spermatozoa plasma membrane integrity. Antioxidant in the extender was crucial for binding free radicals due to freezing-thawing process, thereby maintain spermatozoa plasma membrane integrity (Qamar *et al*., 2023). A high percentage of plasma membrane integrity correlated with lower levels of malondialdehyde in spermatozoa, a finding that aligned with the results was reported (Susilowati *et al*., 2021).

Basil leaf extract (*Ocimum basilicum* L.) was recognized as an effective antioxidant. The flavonoids contained in this extract were known for their natural antioxidant properties, which enabled them to capture free radical molecules and stabilize them by donating electrons. This action inhibited chain reactions that might lead to oxidative stress (Romano *et al*., 2022). Specifically, flavonoids could mitigate lipid peroxidation through a chain-breaking mechanism by capturing ROO– radicals, providing H+ donors, and forming stable complexes with those radicals (Tumilaar *et al*., 2024). In addition to flavonoids, basil leaf extract also provided arginine (Romano *et al*., 2022), an important amino acid that supports spermatozoa quality and motility by promoting the production of NO (Luo *et al*., 2021). Arginine served as a precursor to NO, which was synthesized by the enzyme NOS located in the spermatozoa acrosome, using oxygen and nicotinamide adenine dinucleotide phosphate (Dutta *et al*., 2022). This synthesis allowed NO to diffuse freely across the cell membrane to bind with free radicals. Nitric oxide played a critical role in neutralizing superoxide, a byproduct of spermatozoa metabolism. Elevated levels of superoxide could lead to peroxidation of the spermatozoa's phospholipid membranes, which were essential for proper receptor function and enzymatic activity, ultimately resulting in functional impairment (Kowalczyk *et al*., 2022).

The integrity of the plasma membrane in spermatozoa was crucial for their overall viability. Damage to this membrane could create anisoosmotic conditions, leading to intracellular leakage, which adversely affected ATP breakdown and spermatozoa motility (Fraser *et al*., 2001). Furthermore, the integrity of plasma membranes was vital for spermatozoa metabolism, capacitation, acrosome reaction, and the binding of spermatozoa to the oocyte surface.

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Spermatozoa morphological abnormality

Post-thawed cow semen abnormalities of more than 20% caused a decreased fertility (Susilawati, 2011). Addition of 0.3% basil leaf extract to AndroMed® extender resulted in the lowest (less than 10%) morphological spermatozoa abnormality. The addition of basil leaf extract served as an effective antioxidant primarily due to its flavonoid content. Antioxidants were vital substances that could slow down or prevent oxidation, thereby protecting cellular structures, particularly cell membranes, from damage inflicted by free radicals (Walke *et al*., 2023).

Flavonoids and arginine also played a significant role as an antioxidant. It helped inactivating superoxide anions by promoting the production of NO, which subsequently reduced lipid peroxidation in spermatozoa membranes. The synthesis of NO occurred from arginine through the action of the enzyme NOS, which was found within spermatozoa (Jomova *et al*., 2024). Furthermore, NO was crucial for the defense against the formation of ROS during the freezing process of spermatozoa at -196°C in liquid nitrogen (Malik *et al*., 2015). However, damage to the plasma membrane of spermatozoa could occur due to the high levels of unsaturated fatty acids, which were particularly vulnerable to lipid peroxidation, especially during semen preservation (Khalil *et al*., 2017). An increased incidence of abnormal spermatozoa was often associated with a decline in progressive motility (Chao *et al*., 2023). Generally, a morphological abnormality rate of 8% to 10% did not significantly affect fertility; however, abnormalities exceeding 30% per ejaculate could lead to a decline in fertility that might be difficult to anticipate (Perry *et al*., 2021).

CONCLUSION

Evaluation of spermatozoa motility, viability, spermatozoa plasma membrane integrity, and spermatozoa morphological abnormalities in frozen thawed Simmental bull semen showed that a dose of 0.3% basil leaf extract was most appropriate for improving semen quality in AndroMed® frozen semen extender. Spermatozoa motility (more than 40%) and spermatozoa morphology abnormalities (less than 20%) indicated that post-thawing semen met the requirements for artificial insemination based on the Indonesian National Standard (SNI 4869-1:2021).

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AUTHOR'S CONTRIBUTIONS

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AI and FRS: conceived the idea, designed the mainframe of this manuscript, acquisition, analysis and interpretation of data, AI, FRS, SS and NH: manuscript drafting. EBAH, SPM, KR, TWS, and PS: critically read and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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