# Fresh Semen Characteristics, Biochemical and Mineral Composition of Seminal Plasma from Different Age Groups of Balinese Cattle

**Brilla Widya Witri[1], Iis Arifiantini[2], Wahono Esthi Prasetyaningtyas[3], Ni Wayan Kurniani Karja[2]***

[1]Veterinary Biomedical Science Study Program, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, 16680, Indonesia, [2]Division of Reproduction and Obstetrics, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor 16680, Indonesia, [3]Division of Anatomy, Histology and Embryology, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, 16680, Indonesia.

*Corresponding author: karja_nwk@apps.ipb.ac.id

## Abstract

Age is among the factors affecting reproductive organs, particularly semen characteristics. Semen characteristics are also influenced by composition of seminal plasma that is ejaculated with the sperm. Therefore, this study aimed to investigate characteristics of fresh semen and seminal plasma composition from Balinese cattle aged 3, 8, and 13 years. Semen characteristics observed were concentration, progressive motility, viability, plasma membrane integrity, acrosome integrity, and sperm abnormalities. Biochemical composition of seminal plasma analyzed was fructose, malondialdehyde (MDA), total antioxidant capacity (TAC), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), total protein (TP), albumin, cholesterol, and triglycerides. Mineral compositions observed were Na, K, Ca, Mg, Zn, Cu, Fe, and Se. The results showed that there were no significant differences ($p > 0.05$) in semen characteristics across age groups, meaning that semen of Balinese cattle can still be processed into frozen semen even at an older age, provided it meets the SNI 4869-1:2024 standards. Furthermore, the concentrations of each seminal plasma biochemical component were as follows: fructose (377.5–615.4 mg/dL), MDA (9.7–10.36 nmol/mL), TAC (66.22–67.22%), GOT (203–265 U/L), GPT (19–35 U/L), TP (4.3–5.5 g/dL), albumin (1.7–1.9 g/dL), cholesterol (11–35 mg/dL), and triglycerides (822–1.078 mg/dL). Surprisingly, the concentration of triglycerides in semen plasma in this study was very high compared to those reported in previous studies on several cattle breeds. Seminal plasma mineral concentrations included Na (1.500–1.900 mg/kg), K (600–700 mg/kg), Ca (600–800 mg/kg), Mg (38.85–45.16 mg/kg), Zn (5.33–9.64 mg/kg), Cu (0.06–0.12 mg/kg), Fe (11.85–15.88 mg/kg), and Se (0.92–1.48 mg/kg). These findings indicate that age did not affect semen characteristics, biochemical, and mineral composition of seminal plasma in Balinese cattle.

Keywords: age, Balinese cattle, semen characteristics, seminal plasma composition

**Received:** February 12, 2025 **Revised:** May 7, 2025 **Accepted:** June 13, 2025

## INTRODUCTION

Artificial insemination (AI) is a reproductive technology capable of increasing the population, productivity, and genetic quality of livestock. The successful outcome of AI depends on the quality of semen. Semen consists of sperm and seminal plasma (Juyena and Stelletta, 2012). Sperm are produced in the seminiferous tubules of the testes and mature in the epididymis, but sperm can fertilize *in vivo* after exposure to seminal plasma (Yamanaka *et al*., 2024). Seminal plasma is a mixture of organic and inorganic compounds that serve multiple roles, including acting as carriers, providing nutrients, protecting sperm, and facilitating the elimination of metabolic waste. Bovine seminal plasma is composed of fluid secretions from the testes, epididymis, ampulla, vesicular glands, prostate, and bulbourethral glands. It contains various compounds, such as minerals, proteins, enzymes, lipids, carbohydrates, and antioxidants (Vitku *et al*., 2017). Seminal plasma plays a crucial role in activating sperm and enhancing motility, providing a transportation medium, supplying nutrients, and preventing premature capacitation. This supports the interactions between sperm and oocytes (Juyena and Stelletta, 2012). Some studies have shown that fructose and antioxidants are related to motility (Velho *et al*., 2018; Vince *et al*., 2018). Fe and Zn are associated with motility and morphology (Pipan *et al*., 2021) while Ca, glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) are connected to motility and abnormalities (Shelke and Dhami, 2002; Talluri *et al*., 2017). Cholesterol is related to concentration, while albumin and K are connected to sperm viability (Shelke and Dhami, 2002).

Increased age in cattle causes organ aging in the reproductive system (Vince *et al*., 2018). Aging is a natural process causing irreversible changes influenced by endogenous and environmental factors (Gunes *et al*., 2016). It causes the depletion of germ cells in seminiferous tubules and the apoptosis of epididymal epithelial cells, which interferes with spermatogenesis and sperm maturation (Dong *et al*., 2022; Endo *et al*., 2024). Aging disrupts the hypothalamus–pituitary–testis (HPT) axis, resulting in lower testosterone levels. The composition of seminal plasma is affected by testosterone level and the degree of apoptosis in epithelial cells of accessory glands (Hafizuddin *et al*., 2023).

Previous studies demonstrated a decrease in semen quality with increasing age in Simmental cattle (Baharun *et al*., 2021), Jersey cattle (Sankhi *et al*., 2019), Ongole (Suyadi *et al*., 2020), Ongole, Brahman, and Simmental cattle (Budiyanto *et al*., 2021). Other reports documented that age did not affect semen quality in Balinese cattle (Nabilla *et al*., 2018), Sahiwal cattle (Ahmad *et al*., 2011), and Simmental cattle (Vince *et al*., 2018). Therefore, this study aimed to analyze the semen characteristics, biochemical, and mineral composition of seminal plasma in Balinese cattle during different age groups. Balinese cattle derived from the domestication of bulls (*Bos javanicus*) were used as subjects because of their adaptability to the tropical climate, serving as a suitable option for breeding in Indonesia (Mohamad *et al*., 2009). These cattle have high fertility (80%), low calf mortality, elevated carcass percentage, resistance to several diseases, and the ability to use low-quality feed (Rahmatullaili *et al*., 2019).

## MATERIALS AND METHODS

### Ethical Approval

This study has been approved by the Animal Ethics Commission of the School of Veterinary Medicine and Biosciences, IPB University with number 217/KEH/SKE/VI/2024. The working procedures in this study did not cause any suffering to the animals used. Fresh semen was collected using an artificial vagina, the bulls used in this study were all familiar with the semen collection procedure. Semen was collected by professionals.

### Study Period and Location

Fresh semen samples were taken and analyzed at the Riau Regional Artificial Insemination Center (RAIC). Fructose, malondialdehyde (MDA), and total antioxidant capacity (TAC) analyses were conducted at the Biochemistry Laboratory of FMIPA IPB University. GOT, GPT, total protein (TP), albumin, glucose, cholesterol, and triglyceride analyses were conducted at the Center for Primate Animal Studies, IPB University. Mineral analysis of Na, Ca, K, Mg, Zn, Fe, Cu, and Se was conducted at the Integrated Laboratory of IPB University. The study took place from June–August 2024.

### Experimental Design

In this study, semen was collected by artificial vagina from 9 Balinese cattle, with 3 cattle each aged 3, 8, and 13 years old. Samples were divided for analysis of fresh semen characteristics. During the evaluation, the samples were placed in a waterbath at 37ºC to keep the sperm active. The remaining semen was centrifuged at 6000 rpm for 30 minutes to separate the sperm and seminal plasma. Subsequently, seminal plasma from individuals of the same age group was pooled for seminal plasma composition analysis. The seminal plasma was transported in a cooler box with ice gel (± -10ºC) and stored at -20ºC for further seminal plasma analysis.

**Fresh Semen Analysis**

Progressive motility and sperm concentration were analyzed using computer-assisted sperm analysis (CASA), software (Minitube version 3.5.6.2) (Setiyono *et al*., 2020) with modification. Fresh semen was diluted with warmed Tris buffer (25:725) at 37ºC before analysis. A total of 3 µL of the mixture was placed in a warmed Leja counting chamber at the same temperature. Furthermore, software program was run according to the guidelines and observations were made in 5 fields of view. Sperm viability was evaluated by eosin nigrosine staining (Pardede *et al*., 2020). Semen and eosin nigrosin were mixed at a ratio of 1:4 in an glass slide and homogenized. Serial preparations from the mixture were made, dried on a heating table at 37ºC and observed under a light microscope on 200 sperm cells. Plasma membrane integrity was evaluated via a hypoosmotic swelling test (HOST). Approximately 990 µL of HOST solution comprising 1.35 g of fructose and 0.737 g of Tri-sodium citrate dihydrate in 100 mL of milli-Q water was mixed with 10 µL semen (1:100). The mixture was incubated at 37ºC for 30 minutes. Five µL of the mixture was placed on a warmed glass slide and covered with a cover glass. The preparations were observed using a microscope at 400× magnification, evaluating 200 sperm cells (Putri *et al*., 2023).

Evaluation of acrosome integrity was performed using Giemsa staining. The semen was diluted with NaCl solution (1:4) and the preparations were made, dried, and fixed with methanol for 10 minutes at room temperature. After that, the preparations were stained with Giemsa dye at room temperature for 3 hours, rinsed with running water, dried, and observed under a light microscope at 400× magnification on 200 sperm. Subsequently, an evaluation was conducted on 200 sperm (Prihantoko *et al*., 2022). Giemsa has a low molecular weight and can penetrate the cell membrane and bind to proteins in the acrosome, thus coloring the acrosome. Giemsa staining was chosen because it is a simple and easy method but still able to describe the integrity of the sperm acrosome. Using single Giemsa staining is more efficient and clearer than double staining, such as Trypan Blue Giemsa (TBG) (Prihantoko *et al*., 2022). Sperm abnormality evaluation was evaluated using the Williams staining method (Baharun *et al*., 2021). Briefly, the preparations from a mixture of semen and NaCl solution (1:4) were made, dried, and stained with Williams solution at room temperature. Last, The preparations were observed using a microscope at 400× magnification on 200 sperm cells. This method was chosen because it has a simple procedure. The staining results are clearer than other stains, making evaluating the morphological structure of the head, midpieces, and tail easier. In addition, the staining results have a stable color and can be stored for a long time (Murali *et al*., 2024). All evaluations of fresh semen characteristics were conducted with two replicates on each individual.

**Seminal Plasma Composition Analysis**

Seminal plasma biochemical composition concentrations were analyzed using commercial kits, GOT and GPT (*Labtest*, Brazil), cholesterol, glucose, triglycerides (*Greiner*, Germany), albumin (*Labkit*, Barcelona), and total protein (*Biomaxima*, Poland). The MDA concentration was measured via the thiobarbituric acid reactive species (TBARS) assay method (Subramanian *et al*., 2018). The total antioxidant capacity (TAC) was measured using the 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay (Subramanian *et al*., 2018). Fructose was measured calorimetrically following the method of Trang (2018). The mineral concentrations of Na, K, Ca, Mg, Zn, Cu, Fe, and Se were analyzed by the Atomic Absorption Spectroscopy (AAS) method (Waheed *et al*., 2022).

**Data Analysis**

The obtained data in this study about semen characteristics were analyzed using analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to analyze differences in means between the groups. Differences were considered significant at $p < 0.05$. The biochemical and mineral composition data of seminal plasma presented in table.

## RESULTS AND DISCUSSION

The results of the examination of fresh semen characteristics, such as concentration, progressive motility, viability, plasma membrane integrity, acrosome integrity, and sperm abnormalities, of Balinese cattle in the 3-, 8-, and 13-year-old age groups are presented in Figure 1. The sperm concentration of Balinese cattle aged 3, 8, and 13 years was 111.9 ± 12 x $10^7$, 114.2 ± 19.7 x $10^7$, and 77.97 ± 13 x $10^7$, respectively. The sperm progressive motility in the age groups of 3, 8, and 13 years was 62.78 ± 2.38%, 64.14 ± 6.11%, and 50.75 ± 1.38%. Sperm viability in the 3, 8, and 13-year-old groups was 67.72 ± 4.23%, 70.26 ± 0.79%, and 68.29 ± 3.92%, respectively. The plasma membrane integrity of the 3, 8, and 13-year-old groups was 58.99 ± 6.05%, 63.55 ± 4.56%, and 69.22 ± 6.24%, respectively. Sperm acrosome integrity was 88.32 ± 1.1%; 92.01 ± 1.55%; 90.84 ± 0.88%, respectively. Sperm abnormalities were 9.44 ± 0.55%; 13.82 ± 3.24%; and 21.38 ± 3.85%, respectively. Statistical analysis showed no significant differences ($p > 0.05$) in concentration, progressive motility, viability, plasma membrane integrity, acrosome integrity, or sperm abnormalities across the different age groups.

**Table 1.** Biochemical composition of seminal plasma of Balinese cattle in different age groups

| Age | Fructose mg/dL | MDA nmol/mL | TAC % | GOT U/L | GPT U/L | TP g/dL | Albumin g/dL | Cholesterol mg/dL | Triglyceride mg/dL |
|---|---|---|---|---|---|---|---|---|---|
| 3 | 552.9 | 10.244 | 66.55 | 220 | 33 | 5.5 | 1.7 | 11 | 1078 |
| 8 | 615.4 | 9.699 | 67.22 | 203 | 19 | 4.9 | 1.9 | 35 | 822 |
| 13 | 377.5 | 10.352 | 66.22 | 265 | 35 | 4.3 | 1.7 | 23 | 854 |

MDA = Malondialdehyde, TAC = total antioxidant capacity, GOT = glutamic oxaloacetic transaminase, GPT = glutamic pyruvic transaminase, TP = total protein.

**Table 2.** Mineral composition of Balinese cattle semen plasma at different age groups

| Age | Na mg/kg | K mg/kg | Ca mg/kg | Mg mg/kg | Zn mg/kg | Cu mg/kg | Fe mg/kg | Se mg/kg |
|---|---|---|---|---|---|---|---|---|
| 3 | 1.900 | 600 | 700 | 38.85 | 9.64 | 0.07 | 13.59 | 0.92 |
| 8 | 1.500 | 700 | 800 | 45.16 | 5.33 | 0.12 | 11.85 | 1.48 |
| 13 | 1.700 | 600 | 600 | 39.8 | 5.6 | 0.06 | 15.88 | 1.16 |

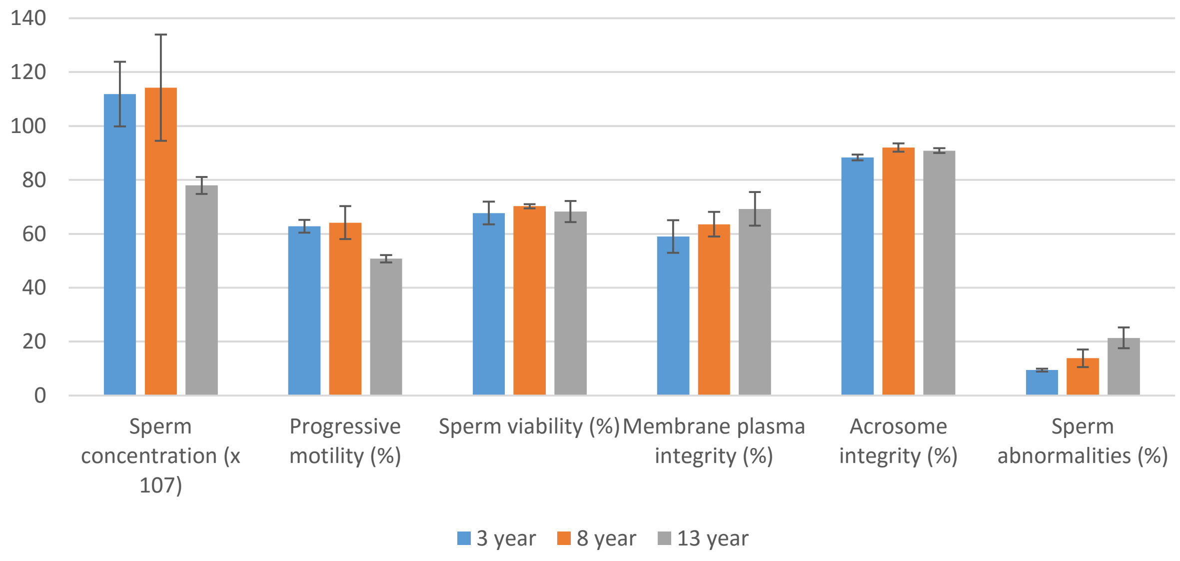


**Figure 1.** Fresh semen characteristics of Balinese cattle in different age groups.

The biochemical composition of seminal plasma from 3-, 8-, and 13-year-old Balinese cattle is shown in Table 1. The fructose concentration in this study ranged from 377.5 to 615.4 mg/dL. The concentrations of MDA, TAC, TP, and albumin in all age groups did not differ much. The GOT and GPT were lower in the 8-year-old group than in others. The cholesterol concentration was the highest at 8 years, while triglycerides were the lowest in that age group. Seminal plasma mineral concentrations of Balinese cattle are presented in Table 2. There were variations in mineral concentrations in age groups, but the differences were within a narrow range of values.

Semen characteristics must be evaluated due to their influence on the natural fertilization process (*in vivo*). Fertilization will only occur when the sperm can meet the ovum at the right place, time, and conditions to fuse with the egg. Therefore, fresh sperm must have a normal morphology, an intact plasma membrane, and acrosome, be viable, and have sufficient motility to reach the fertilization site (Yamanaka *et al*., 2024). This study found no differences ($p > 0.05$) in semen characteristics, such as concentration, progressive motility, viability, plasma membrane integrity, acrosome integrity, and sperm abnormalities among Balinese cattle aged 3, 8, and 13 years. This finding is consistent with previous studies on Balinese cattle by (Bayu and Isnaini, 2020) and (Nabilla *et al*., 2018) that found age did not affect the quality of semen produced. Some cattle still produced semen of unchanged quality in old age (Gupta *et al*., 2025). Maturation and aging occur at different ages, depending on the breed. Since the reproductive peak differs for each breed, age-related decline in reproduction is also different (Snoj *et al*., 2013). Balinese cattle start puberty at 9 months of age, after which semen quantity and quality increase until 2 years of age. Semen at age of >2 years old can be produced continuously (Suyadi *et al*., 2020). The youngest cattle used in this study were 3 years old and their semen production was stable in quantity and quality, without being significantly different from others. Balinese cattle produce semen with the best characteristics between 7 and 12 years old (Sitanggang *et al*., 2020). The effect of aging on males occurs slowly and does not change drastically immediately (Ottinger, 2010). Therefore, semen samples from 13-year-old Balinese cattle were not different from the other groups. Previous study also reported that older Indonesian local cattle showed better motility and concentration than Simmental cattle (Novianti *et al*., 2020).

Seminal plasma is the transport medium for sperm and contains important factors for fertilization. Aging can affect function of somatic tissues, including the epithelium of the reproductive tract and accessory glands due to low testosterone levels, oxidative stress on the accessory glands, and apoptosis of the glandular epithelium (Elzanaty, 2007). The concentrations of seminal plasma component, such as proteins, cholesterol, and triglycerides are regulated locally by epithelial cells along the reproductive organs and accessory glands, which are influenced by aging (Vince *et al*., 2018). Aging process of accessory glands also occurs slowly, depending on the race and health of the individual (Moula and Amiri, 2022). Furthermore, seminal plasma composition depends on each individual due to significant variation in the same animal with an identical husbandry system (Moula and Amiri, 2022).

Examination conducted in seminal plasma showed that fructose tended to be higher at age 8 than other groups. Aging reduces Leydig cell numbers in the testes, leading to lower testosterone levels, which in turn decreases fructose concentration in seminal plasma (Velho *et al*., 2018). Juyena and Stelletta (2012) reported that fructose concentrations in bulls were generally 150–900 mg/dL. The fructose concentrations of all age groups in this study were within the range of values. Fructose is known as the primary source of energy that is used through the glycolysis pathway. This shows that the fructose concentration significantly affects the energy produced. A lack of energy will profoundly affect sperm metabolism and reduce motility (Velho *et al*., 2018).

The concentrations of TP and albumin were similar between age groups (Vince *et al*., 2018).

Total protein is positively related to sperm quality and interacts with the epithelium of the female reproductive tract for successful fertilization. Meanwhile, total seminal plasma protein is derived from the secretions of the testes, epididymis, and accessory glands. They are also present in all stages of sperm, such as transportation, protection, and maturation. According to Samanta *et al*. (2018), proteins from seminal plasma bind to the sperm surface through exosomes, regulate function, and interact with the female reproductive tract. Based on this role, total protein can affect sperm fertility. One of the proteins observed is albumin secreted by the testes, epithelium of the caudal epididymis, and prostate (Elzanaty, 2007). Seminal plasma albumin plays a role in transporting molecules to the sperm plasma membrane during the maturation process in the epididymis, the acrosome reaction, and sperm–oocyte fusion (Arroteia *et al*., 2014). Albumin is a non-enzymatic antioxidant that can bind to fatty acids to prevent their oxidation. It also binds to $Cu^{2+}$ or $Fe^{2+}$ ions to prevent the formation of free radicals (Roche *et al*., 2008; Sangen *et al*., 2021).

The cholesterol concentrations in this study were highest in the 8-year age group. The cholesterol concentrations in this study were consistent with a previous study reported that cholesterol concentration in Simmental cattle was greater in the 5–10 years age that 2–4 years age group (Beer-Ljubić *et al*., 2009). Generally, cholesterol is required to maintain the integrity and fluidity of cell membranes. It also plays an active role in the structure and function, metabolism, capacitation, and fertilization of sperm (Juyena and Stelletta, 2012). Cholesterol in seminal plasma will be replaced in plasma membrane to maintain sperm function and delay capacitation to occur at the right time. Cholesterol is required in spermatogenesis and protects sperm after ejaculation (Neergaard *et al*., 2018). A reduced cholesterol level in the plasma membrane initiates sperm capacitation (Beer-Ljubić *et al*., 2009).

The triglyceride concentration was higher in the 3-year age group compared to other age groups. This result was similar to Kelso *et al*. (1997), where the triglycerides were relatively high at 2–3 years of age. Different results were reported by Gafer *et al*. (2015) who found that triglyceride concentrations in beef cattle and buffalo were previously reported to be 54.8 – 63.45 mg/dL and 50.79 – 58.32 mg/dL, respectively. An increase in triglycerides within these ranges showed better sperm motility and plasma membrane integrity (Gafer *et al*., 2015). The triglyceride concentration in this study was very high approximately >10 times higher than the report by Gafer *et al*. (2015). In addition to fructose, triglycerides are an energy source for sperm which is generated through the β-oxidation pathway in sperm mitochondria (Tirpák *et al*., 2022). Hypertriglyceridemia decreases sperm motility and viability while increasing sperm apoptosis. Additionally, hypertriglyceridemia causes changes in plasma membrane composition, reducing the fluidity and integrity of the sperm plasma membrane (Zubi and Alfarisi, 2021).

The levels of MDA and TAC in this study were not significantly different among the 3-, 8-, and 13-year-old age groups. This result was in line with the report by Ahmed *et al*. (2018), where MDA and TAC were independent of age. Dwinofanto *et al*. (2019) found that MDA concentration in Balinese cattle was 5.4 nmol/mL. MDA is a biomarker of lipid peroxidation, which is the end product of oxidative stress to fatty acids. The results obtained have a greater range than previous studies on Balinese cattle. The high MDA concentration in seminal plasma is due to elevated lipid peroxidation and a lack of antioxidant levels (Agarwal *et al*., 2016). Semen plasma antioxidants are derived from accessory organ secretions. During spermatogenesis and maturation, sperm depend on antioxidants produced by the testes and epididymis. Subramanian *et al*. (2018) stated that TAC values <77.4% impair sperm function, particularly membrane integrity. The TAC concentrations in all age groups in this study were low, this reduction led to high MDA. These high MDA and low TAC values contribute to suboptimal sperm conditions, as lipid peroxidation causes cell damage, increased plasma membrane permeability, and decreased motility, viability, fluidity, plasma membrane integrity, and overall sperm quality (Ahmed *et al*., 2018).

In this study, seminal plasma GOT and GPT concentrations were higher compared to the previous report by Jalmeria *et al*. (2020). In addition, Atroshchenko *et al*. (2019) indicated that GOT and GPT were not significantly different in seminal plasma of horses in different ages. Both GOT and GPT are intracellular transaminase enzymes, which can leak into the seminal plasma when the sperm is damaged. The increase in GOT and GPT enzymes in plasma results from abnormal sperm, cytoplasmic droplets, damage of sperm plasma membrane, affect reproductive tract epithelia (El-Sharawy *et al*., 2017). The study by Gupta and Singh (2019) on semen plasma from Jersey and Sahiwal x FH cattle showed relatively high concentrations of GOT and GPT in low sperm quality. GOT and GPT activities are in line with each other and are directly proportional, showing the same quality in seminal plasma (Jalmeria *et al*., 2020).

A certain amount of mineral is essential for normal growth, reproduction, and immunity, so the abundance these minerals in seminal plasma affects male fertility dynamics (Schmid *et al*., 2013). Mineral concentrations in seminal plasma of the 3-, 8- and 13-year-old groups were similar in this study. Schmid *et al*. (2013) also reported that there are no significant differences in the concentrations of K, Ca, Fe, Cu, and Zn in semen plasma across various age groups. Eissa *et al*. (1992) reported that Na, K, Ca, Mg, Fe, and Zn concentrations were not significantly different in buffaloes of different age groups. Similar report was documented by Khan *et al*. (2015) regarding the concentrations of Na and K. Seminal plasma mineral concentrations depended on intake through feed or supplements, intestinal absorption, and mineral carrier proteins (Giacconi *et al*., 2017). Minerals must be absorbed by epithelial cells of the gastrointestinal tract, particularly the small intestine. These minerals enter the lamina propria paracellularly and transcellularly to be passed on to the blood vessels. Some metals bind to carrier proteins in intra- and extracellular body fluids (Goff, 2018). In semen plasma, minerals are secreted by glands along the reproductive tract, with varying predominance. Zn is mainly secreted from the prostate gland, serving as a marker of prostate conditions (Fallah *et al*., 2018). Ca and Cu are mainly secreted from the vesicular glands. Meanwhile, Na, K, and Mg are secreted from bulbourethral glands, and Fe by the prostate (Eissa *et al*., 1992).

Mineral concentrations of Na and K in this study are similar to the report by Zulyazaini *et al*. (2016) in Aceh cattle. Na and K minerals affect the osmotic balance and osmolality of semen plasma, which plays an important role in sperm cell activation (Asadpour, 2012). Na plays a role in maintaining the intracellular pH balance (Zulyazaini *et al*., 2016). Ca in seminal plasma affects each physiological stage of sperm, including motility, metabolism, acrosome reaction, and fertilization, as sufficient amount is required for the capacitation process. High intracellular Ca concentration will activate adenylate cyclase, increasing intracellular cAMP and inducing sperm hyperactivation (Zulyazaini *et al*., 2016; Hidayatik *et al*., 2021). Additionally, Ca functions as an intracellular messenger crucial for sperm motility, hyperactivation, capacitation, acrosome reaction, and chemotaxis in the female reproductive tract (Mirnamniha *et al*., 2019).

Several minerals including Zn, Cu, Fe, Mg, and Se are essential for the enzyme production. In this study, the Zn concentration was similar to results reported in Jersey cattle by Sood *et al*. (2020), while Cu was not substantially different from Tirpák *et al*. (2022). The Se concentration was similar to the value was reported by Khaki *et al*. (2021) in simental cattle. Mg, Zn, Cu, Fe, and Se play essential roles in antioxidant enzymes formation. Cu and Zn bind to antioxidant enzymes that convert superoxide radicals to $H_2O_2$ in the cytosol. Fe is responsible for breaking down $H_2O_2$ directly into oxygen and water (Tvrdá *et al*., 2012). Concerning ATP production, Cu acts as cofactor of mitochondrial cytochrome-C-oxidase (Ferrer *et al*., 2024). Mg is an intracellular mineral that binds ATP (MgATP), functioning in mediating ADP ribosylation and inhibiting endonuclease activity essential to protect DNA

(Eghbali *et al*., 2010). In energy metabolism, Zn is included through sorbitol and lactate dehydrogenase (Ferrer *et al*., 2024). Zn is also important for germ cell proliferation and is antibacterial (Fallah *et al*., 2018). Meanwhile, deficiencies of Zn, Cu, and Se in plasma will decrease sperm concentration, antioxidant capacity, longevity, motility, and viability, and increase free radical and sperm abnormalities (Ferrer *et al*., 2024). This shows that high mineral concentration above maximum threshold is toxic and reduces sperm motility (Katayose *et al*., 2004). Excess minerals bind with $H_2O_2$ to form free radicals that reduce motility, viability, and antioxidant capacity, increasing abnormality (Ferrer *et al*., 2024).

## CONCLUSION

This study showed that age did not affect characteristics of fresh semen or biochemical and mineral composition of seminal plasma in Balinese cattle. This finding illustrates that Balinese cattle semen can still be produced into frozen semen as long as the quality of the semen used is according to SNI standards. The finding of very high triglyceride concentrations in all age groups is of note. We suggest further research on how this occurs and what the optimal value of triglyceride concentration in semen plasma is that supports sperm characteristics.

## ACKNOWLEDGEMENTS

This study was funded by the Directorate General of Higher Education, Research, and Technology, Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia, through the PMDSU research scheme under the contract for the implementation of the research program in 2024 (No : 027/E5/PG.02.00.PL/2024). The authors are grateful to the Riau Regional Artificial Insemination Center for their invaluable assistance and facilitating this study.

## AUTHORS' CONTRIBUTIONS

BWW: Conceptualization, Methodology, Writing original Draft. IA: Conceptualization, Validation, Formal analysis, Data curation, Review & Editing, and supervision. WEP: Conceptualization, Formal analysis, Review & editing, Supervision. NWKK: Conceptualization, validation, formal analysis, review & editing, supervision.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

## REFERENCES

Agarwal, A., Roychoudhury, S., Bjugstad, K. B., & Cho, C. L. (2016). Oxidation-reduction potential of semen: what is its role in the treatment of male infertility?. *Therapeutic Advances in Urology*, 8(5), 302–318.

Ahmad, E., Ahmad, N., Naseer, Z., Aleem, M., Khan, M. S., Ashiq, M., & Younis, M. (2011). Relationship of age to body weight, scrotal circumference, testicular ultrasonograms, and semen quality in Sahiwal bulls. *Tropical Animal Health and Production*, 43(1), 159–164.

Ahmed, S., Khan, M. I.-R., Ahmad, M., & Iqbal, S. (2018). Effect of age on lipid peroxidation of fresh and frozen-thawed semen of Nili-Ravi buffalo bulls. *Italian Journal of Animal Science*, 17(3), 730–735.

Arroteia, K. F., Barbieri, M. F., Souza, G. H. M. F., Tanaka, H., Eberlin, M. N., Hyslop, S., Alvares, L. E., & Pereira, L. A. V. D. (2014). Albumin Is Synthesized in Epididymis and Aggregates in a High Molecular Mass Glycoprotein Complex Involved in Sperm-Egg Fertilization. *PLoS ONE*, 9(8), 1–11.

Asadpour, R. (2012). Relationship between mineral composition of seminal plasma and semen quality in various ram breeds. *Acta Scientiae Veterinariae*, 40(2), 1027–1035.

Atroshchenko, M. M., Kudlaeva, A. M., Fomina, M. A., Kalashnikov, V. V, Zaitcev, A. M., Denisova, O. V, Navasardyants, D. G., Belonovskaya, O. S., & Pasko, A. A. (2019). Analysis of seminal plasma biochemical parameters and sperm cryostability in different age groups of stallions. *IOP Conference Series: Earth and Environmental Science*, 341(1), 1–7.

Baharun, A., Said, S., Arifiantini, R. I., & Karja, N. W. K. (2021). Correlation between age, testosterone and adiponectin concentrations, and sperm abnormalities in Simmental bulls. *Veterinary World*, 14(8), 2124–2130.

Beer-Ljubić, B., Aladrović, J., Marenjak, T. S., Laskaj, R., Majić-Balić, I., & Milinković-Tur, S. (2009). Cholesterol concentration in seminal plasma as a predictive tool for quality semen evaluation. *Theriogenology*, 72(8), 1132–1140.

Budiyanto, A., Arif, M., Alfons, M. P. W., Fani, R. T., Hafid, A. F., Wicaksono, B., Insani, K. M., & Herdinta, M. (2021). The Effect of Age and Breed on The Quality of Bull Semen in The Regional Artificial Insemination Centre. *Acta Veterinaria Indonesiana*, Spesial Issues, 132–136.

Dong, S., Chen, C., Zhang, J., Gao, Y., Zeng, X., & Zhang, X. (2022). Testicular aging, male fertility and beyond. *Frontiers in Endocrinology*, 13, 1012119–1012134.

Dwinofanto, H., Rimayanti, R., Mustofa, E., Susilowati, S., & Hernawati, T. (2019). The effect of duration of preservation on the quality, MDA level, and DNA damage of post-thawed Bali cattle bull sperm. *Iraqi Journal of Veterinary Sciences*, 32(2), 249–252.

Eghbali, M., Mortaza Alavi-Shoushtari, S., Asri-Rezaei, S., Khadem Ansari, M.-H., & Morteza Alavi-Shoushtari, S. (2010). Calcium, magnesium and total antioxidant capacity (TAC) in seminal plasma of water buffalo (*Bubalus bubalis*) bulls and their relationships with semen characteristics. *Veterinary Research Forum,* 1(1), 12–20.

Eissa, H. M., El-Tohamy, M. M., & Abdou, M. S. S. (1992). Bulk and trace elements in the accessory glands of buffalo bulls. *British Veterinary Journal*, 148(6), 557–565.

El-Sharawy, M., Eid, E., Darwish, S., Abdel-Razek, I., Islam, M. R., Kubota, K., Yamauchi, N., & El-Shamaa, I. (2017). Effect of organic and inorganic selenium supplementation on semen quality and blood enzymes in buffalo bulls. *Animal Science Journal*, 88(7), 999–1005.

Elzanaty, S. (2007). Association between age and epididymal and accessory sex gland function and their relation to sperm motility. *Archives of Andrology*, 53(3), 149–156.

Endo, T., Kobayashi, K., Matsumura, T., Emori, C., Ozawa, M., Kawamoto, S., Okuzaki, D., Shimada, K., Miyata, H., Shimada, K., Kodani, M., Ishikawa-Yamauchi, Y., Motooka, D., Hara, E., & Ikawa, M. (2024). Multiple ageing effects on testicular/epididymal germ cells lead to decreased male fertility in mice. *Communications Biology*, 7(1), 16–32.

Fallah, A., Mohammad-Hasani, A., & Colagar, A. H. (2018). Zinc is an essential element for male fertility: A review of zn roles in men's health, germination, sperm quality, and fertilization. In *Journal of Reproduction and Infertility*, 19(2), 69–81.

Ferrer, M., Palomares, R., & Maldonadox-Estrada, J. (2024). Role of trace minerals in bull reproductive physiology and semen quality. *Clinical Theriogenology*, 16, 1–7.

Gafer, J., ElRahman, G., & M, R. (2015). Association of Hsp70 Gene Polymorphism And Bull Semen Quality In Winter And Summer Seasons. *Alexandria Journal of Veterinary Sciences*, 46(1), 1–14.

Giacconi, R., Costarelli, L., Piacenza, F., Basso, A., Rink, L., Mariani, E., Fulop, T., Dedoussis, G., Herbein, G., Provinciali, M., Jajte, J., Lengyel, I., Mocchegiani, E., & Malavolta, M. (2017). Main biomarkers associated with age-related plasma zinc decrease and copper/zinc ratio in healthy elderly from ZincAge study. *European Journal of Nutrition*, 56(8), 2457–2466.

Goff, J. P. (2018). Invited review: Mineral absorption mechanisms, mineral interactions that affect acid–base and antioxidant status, and diet considerations to improve mineral status. *Journal of Dairy Science*, 101(4), 12763–2816.

Gunes, S., Hekim, G. N. T., Arslan, M. A., & Asci, R. (2016). Effects of aging on the male reproductive system. In *Journal of Assisted Reproduction and Genetics,* 33(4), 441–454.

Gupta, S., & Singh, M. (2019). Biochemical and functional parameters of freezable and non-freezable ejaculates of bull semen and their correlation. *Indian Veterinary Journal*, 96(4), 47–49.

Gupta, V. K., Mohanty, T. K., Bhakat, M., Kumaresan, A., Baithalu, R. K., Kumar, N., Dewry, R. K., Nain, D., Yadav, R., Arunkumar, R., & Soe, A. (2025). Effect of age-associated oxidative stress on sperm viability, acrosomal integrity and sperm apoptosis and DNA fragmentation in Sahiwal breeding bulls. *Veterinary Research Communications*, 49(71), 71–82.

Hafizuddin, H., Husnurrizal, H., Siregar, T. N., Eriani, K., Wahyuni, S., Ahsan, M. M., Sutriana, A., Anwar, A., & Aliza, D. (2023). Amelioration of Seminal Plasma Testosterone Concentration in Gembrong Goats after In Vivo Administration of PGF2α. *Jurnal Medik Veteriner*, 6(2), 256–261.

Hidayatik, N., Purnomo, A., Fikri, F., & Purnama, M. T. E. (2021). Amelioration on oxidative stress, testosterone, and cortisol levels after administration of Vitamins C and E in albino rats with chronic variable stress. *Veterinary World*, 14(1), 137–143.

Jalmeria, N. S., Panth, S., Pandita, S., Roy, A. K., Ashutosh, M., Mohanty, T. K., Bhakat, M., Punetha, M., & Gupta, D. (2020). Seasonal variations in hormones and enzymes of seminal plasma and its relationship with semen quality in crossbred cattle bulls. *Biological Rhythm Research*, 51(4), 633–643.

Juyena, N. S., & Stelletta, C. (2012). Seminal plasma: An essential attribute to spermatozoa. *Journal of Andrology*, 33(4), 536–551.

Katayose, H., Shinohara, A., Chiba, M., Yamada, H., Tominaga, K., Sato, A., & Yanagida, K. (2004). Effects of Various Elements in Seminal Plasma on Semen Profiles. *Journal of Mammalian Ova Research*, 21(3), 141–148.

Kelso, K. A., Redpath, A., Noble, R. C., & Speake, B. K. (1997). Lipid and antioxidant changes in spermatozoa and seminal plasma throughout the reproductive period of bulls. *Journal of Reproduction and Fertility*, 109(1), 1–6.

Khaki, A., Araghi, A., Lotfi, M., & Nourian, A. (2021). Differences between some biochemical components in seminal plasma of first and second ejaculations in dual-purpose simmental (Fleckvieh) bulls and their relationships with semen quality parameters. *Veterinary Research Forum*, 12(1), 39–46.

Khan, A., Yasinzai, M. M., & Kakar, M. A. (2015). Biochemical analysis of bovine (*Bos indicus*) seminal plasma. *International Journal of Advanced Biological and Biomedical Research*, 3(4), 361–369.

Mirnamniha, M., Faroughi, F., Tahmasbpour, E., Ebrahimi, P., & Beigi Harchegani, A. (2019). An overview on role of some trace elements in human reproductive health, sperm function and fertilization process. *Reviews on Environmental Health*, 34(4), 339–348.

Mohamad, K., Olsson, M., van Tol, H. T. A., Mikko, S., Vlamings, B. H., Andersson, G., Rodríguez-Martínez, H., Purwantara, B., Paling, R. W., Colenbrander, B., & Lenstra, J. A. (2009). On the origin of Indonesian cattle. *PLoS ONE*, 4(5), e5490–e5496.

Moula B, A., & El Amiri, B. (2022). Factors influencing seminal plasma composition and its relevance to succeed sperm technology in sheep: An updated review. *Small Ruminant Research*, 215(2022), 106759–106769.

Murali, P., Muddha, S. S., & Gali, V. (2024). Comparison of special stains for analysing the morphology of sperm: A cross-sectional study. *National Journal of Laboratory Medicine*, 13(1), 1–4.

Nabilla, A., Arifiantini, R. I., & Purwantara, B. (2018). Kualitas Semen Segar Sapi Bali

Umur Produktif dan Non-produktif serta Penentuan Konsentrasi Krioprotektan dalam Pengencer Tris Kuning Telur. *Jurnal Veteriner*, 19(2), 242–250.

Neergaard, R. D., Nielsen, J. E., Jørgensen, A., Toft, B. G., Goetze, J. P., & Jørgensen, N. (2018). Positive association between cholesterol in human seminal plasma and sperm counts: results from a cross-sectional cohort study and immunohistochemical investigations. *Andrology*, 6(6), 817–828.

Novianti, I., Purwantara, B., Herwijanti, E., Nugraha, C. D., Putri, R. F., Furqon, A., Septian, W. A., Rahayu, S., Nurgiartiningsih, V. M. A., & Suyadi, S. (2020). Effect of breeds on semen characteristics of aged bulls in the Indonesian National Artificial Insemination Center. *Jurnal Ilmu-Ilmu Peternakan*, 30(2), 173–179.

Ottinger, M. A. (2010). Mechanisms of reproductive aging: conserved mechanisms and environmental factors. *Annals of the New York Academy of Sciences*, 1204, 73–81.

Pardede, B. P., Agil, M., Yudi, Y., & Supriatna, I. (2020). Relationship of frozen-thawed semen quality with the fertility rate after being distributed in the Brahman Cross Breeding Program. *Veterinary World*, 13(12), 2649–2657.

Pipan, M. Z., Petra, Z., Strajn, B. J., Vrtač, K. P., Knific, T., & Mrkun, J. (2021). Macro- and microelements in serum and seminal plasma as biomarkers for bull sperm cryotolerance. *Acta Veterinaria Scandinavica*, 63(1), 25–34.

Prihantoko, K. D., Kusumawati, A., Pangestu, M., Widayati, D. T., & Budiyanto, A. (2022). Influence of Intracellular Reactive Oxygen Species in Several Spermatozoa Activity in Indonesian Ongole Bull Cryopreserved Sperm. *American Journal of Animal and Veterinary Sciences*, 17(1), 11–18.

Putri, F., Karja, N. W. K., Setiadi, M. A., & Kaiin, E. M. (2023). Influence of Sperm Number and Antioxidant Melatonin in Extender on the Quality of Post-Thawing Sheep Spermatozoa. *Jurnal Ilmu Ternak Dan Veteriner*, 28(1), 863–870.

Rahmatullaili, S., Fatmawati, D., Nisa, C., Winaya, A., Chamisijatin, L., & Hindun, I. (2019). Genetic Diversity of Bali Cattle: Cytochrome b Sequence Variation. *IOP Conference Series: Earth and Environmental Science*, 276(1), 1–9.

Roche, M., Rondeau, P., Singh, N. R., Tarnus, E., & Bourdon, E. (2008). The antioxidant properties of serum albumin. *FEBS Letters*, 582(13), 1783–1787.

Samanta, L., Parida, R., Dias, T. R., & Agarwal, A. (2018). The enigmatic seminal plasma: a proteomics insight from ejaculation to fertilization. *Reproductive Biology and Endocrinology*, 16(1), 41.

Sangen, O. R., Firmawati, A., Marhendra, A. P. W., & Titisari, N. (2021). Spermatogenesis Duration on Adult Javan Langur (*Trachypithecus auratus*) Based on Testosteron Hormone and Luteinizing Hormone (LH). *Jurnal Medik Veteriner*, 4(1), 110–117.

Sankhi, S., Sapkota, K. R., & Regmi, B. (2019). Effect of Age and Frequency of Collection on Quality of Jersey Bulls Semen at National Livestock Breeding Center (NLBC), Nepal. *International Journal of Applied Sciences and Biotechnology*, 7(1), 88–95.

Schmid, T. E., Grant, P. G., Marchetti, F., Weldon, R. H., Eskenazi, B., & Wyrobek, A. J. (2013). Elemental composition of human semen is associated with motility and genomic sperm defects among older men. *Human Reproduction*, 28(1), 274–282.

Setiyono, A., Agus Setiadi, M., Mulyawati Kaiin, E., & Wayan Kurniani Karja, N. (2020). Pola Gerakan Spermatozoa Sapi setelah Diinkubasi dalam Media Fertilisasi dengan Imbuhan Heparin dan/atau Kafein. *Jurnal Veteriner*, 21(3), 458–469.

Shelke, V. B., & Dhami, A. J. (2002). Interrelationships of seminal plasma biochemical, enzymatic and macro-micro mineral profiles with physical attributes and freezability of semen in Jafarabadi bulls.

*Indian Journal of Animal Sciences*, 72(5), 386–390.

Sitanggang, G., Arifiantini, R. I., & Jakaria, J. (2020). Genetic and Non-Genetic Effects on Semen Characteristics of Bali Cattle (*Bos javanicus*). *Jurnal Ilmu Ternak Dan Veteriner*, 25(4), 147–152.

Snoj, T., Kobal, S., & Majdic, G. (2013). Effects of season, age, and breed on semen characteristics in different Bos taurus breeds in a 31-year retrospective study. *Theriogenology*, 79(5), 847–852.

Sood, P., Sharma, A., Chahota, R., & Bansal, S. (2020). Evaluation of certain minerals and seminal plasma proteins in Jersey bulls having major sperm morphological defects. *Indian Journal of Animal Research*, 54(1), 6–10.

Subramanian, V., Ravichandran, A., Thiagarajan, N., Govindarajan, M., Dhandayuthapani, S., & Suresh, S. (2018). Seminal reactive oxygen species and total antioxidant capacity: Correlations with sperm parameters and impact on male infertility. *Clinical and Experimental Reproductive Medicine*, 45(2), 88–93.

Suyadi, S., Herwijanti, E., Septian, W. A., Furqon, A., Nugroho, C. D., Putri, R. F., & Novianti, I. (2020). Some Factors Affecting the Semen Production Continuity of Elite Bulls: Reviewing Data at Singosari National Artificial Insemination Center (SNAIC), Indonesia. *IOP Conference Series: Earth and Environmental Science*, 478(1), 1–5.

Suyadi, S., Purwantara, B., Furqon, A., Septian, W. A., Novianti, I., Nursita, I. W., Nugraha, C. D., Putri, R. F., Pratiwi, H., & Herwiyati, E. (2020). Influences of bull age and season on sperm motility, sperm concentration, and ejaculate volume of Ongole Grade cattle in Singosari National Artificial Insemination Center. *Journal of the Indonesian Tropical Animal Agriculture*, 45(4), 1–6.

Talluri, T. R., Mal, G., & Ravi, S. K. (2017). Biochemical components of seminal plasma and their correlation to the fresh seminal characteristics in Marwari stallions and Poitou jacks. *Veterinary World*, 10(2), 214–220.

Tirpák, F., Halo, M., Tomka, M., Slanina, T., Tokárová, K., Błaszczyk-Altman, M., Dianová, L., Ivanič, P., Kirchner, R., Greń, A., Lukáč, N., & Massányi, P. (2022). Sperm Quality Affected by Naturally Occurring Chemical Elements in Bull Seminal Plasma. *Antioxidants*, 11(9), 1796.

Trang, N. T. (2018). Complete the Manufacturing Procedure of Kit for Determination of Fructose Concentration in Seminal Fluid for Diagnosis Male Infertility. *Biomedical Journal of Scientific & Technical Research*, 8(1), 1–7.

Tvrdá, E., Kňažická, Z., Lukáčová, J., Schneidgenová, M., Massányi, P., Goc, Z., Stawarz, R., & Lukáč, N. (2012). Relationships between iron and copper content, motility characteristics and antioxidant status in bovine seminal plasma. *Journal of Microbiology*, 2(2), 536–547.

Velho C, A. L., Menezes, E., Dinh, T., Kaya, A., Topper, E., Moura, A. A., & Memili, E. (2018). Metabolomic markers of fertility in bull seminal plasma. *PLoS ONE*, 13(4), 1–20.

Vince, S., Žura Žaja, I., Samardžija, M., Majić Balić, I., Vilić, M., Duričić, D., Valpotić, H., Marković, F., & Milinković-Tur, S. (2018). Age-related differences of semen quality, seminal plasma, and spermatozoa antioxidative and oxidative stress variables in bulls during cold and warm periods of the year. *Animals*, 12(3), 559–568.

Vitku, J., Kolatorova, L., & Hampl, R. (2017). Occurrence and reproductive roles of hormones in seminal plasma. *Basic and Clinical Andrology*, 27(1), 19–31.

Waheed, M. M., Meligy, A., Alhaider, A. K., & Ghoneim, I. M. (2022). Relation of seminal plasma trace mineral in the Arabian stallion’s semen with the semen characteristics and subsequent fertility. *Heliyon*, 8(10), 1–7.

Yamanaka, T., Xiao, Z., Tsujita, N., Awad, M., Umehara, T., & Shimada, M. (2024). Testosterone-Induced Metabolic Changes in

Seminal Vesicle Epithelial cells Alter Plasma Components to Enhance Sperm Fertility. *eLife*, 13, 1–62.

Zubi, Z. B. H., & Alfarisi, H. A. H (2021). Hyperlipidemia and male infertility. *Egyptian Journal of Basic and Applied Sciences*, 8(1), 385–396.

Zulyazaini, Z., Dasrul, D., Wahyuni, S., Akmal, M., & Abdullah, Mohd. A. N. (2016). Karakteristik Semen dan Komposisi Kimia Plasma Seminalis Sapi Aceh yang Dipelihara di BIBD Saree Aceh Besar. *Jurnal Agripet*, 16(2), 121–130.

***