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# Detection of Plasma Membrane Alpha Enolase (ENO1) and Its Relationship with Sperm Quality of Bali Cattle

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## ABSTRACT

**Bali cattle are the indigenous cattle well known attributed to their high fertility. Various proteomes in sperm are substances that can determine sperm quality and fertility. One of which is alpha enolase (ENO1). This study aims to assess the presence of ENO1 in the plasma membrane of sperm and its relationship with the semen quality of Bali cattle. The study used 30 ejaculates from 5 bulls for sperm quality assessment and detection of ENO1. Sperm quality is indicated by total motility, progressive motility, kinematics motility, viability, plasma membrane integrity, and ENO1 quantity. Sperm motility and kinematics motility were measured using CASA, while viability was assessed by eosin-nigrosine differential staining. The HOS test was used to determine plasma membrane integrity, and ENO1 quantity was measured by the ELISA method. Data were analyzed using ANOVA (randomized group design), linear regression, Pearson's correction, and the t-Student test. The results showed that ENO1 was detected in the plasma membrane at 1.27 ng/$10^6$ sperms. The amount of alpha-enolase in the plasma membrane of Bali cattle sperm is affected by sperm concentration ($p<0.01$) and not involved in sperm motility. There was no correlation between plasma membrane ENO1 quantity and semen quality. The results of this experiment indicated that alpha-enolase in the plasma membrane of Bali cattle sperm is affected by sperm concentration but not related to sperm quality.**

***Keywords:** ENO1; plasma membrane; proteomes; sperm quality*

## INTRODUCTION

Bali cattle (*Bos javanicus*) is the result of wild cattle (*Bibos banteng*) domestication that has spread evenly throughout Indonesia (Purwantara *et al.*, 2012). Bali cattle have high adaptability and fertility (Juliana *et al.*, 2015). The reproductive capacity of cattle is not only determined by female fertility but also by male fertility. One factor determining male fertility is the ability of sperm to fertilize the oocyte. The capacity of sperm is determined by many factors, including the presence of proteins (proteomes), both in seminal plasma and sperm (Kastelic & Thundathil, 2008; D'Amours *et al.*, 2010).

Before ejaculation, bovine sperm are coated with proteins produced by the epididymis and accessory sex glands (Fair & Lonergan, 2018). The presence of protein can determine sperm capacity. Protein is an essential supporting substance for physiological sperm function (Pérez-Patiño *et al.*, 2019). The role of proteins in determining sperm fertility is related to the process and mechanism of energy requirement compliance, plasma membrane function, sperm-oocyte interaction, and cell cycle regulation. Proteins also have a role in post-translational changes during maturation, capacitation, and protection from oxidative stress (Gaviraghi *et al.*, 2010; Park *et al.*, 2012). Proteins present in seminal plasma and sperm have been shown to correlate with sperm motility, morphology, and fertility (D'Amours *et al.*, 2010). The protein content in sperm varies depending on the male's type, breed, and fertility level (Dixit *et al.*, 2016).

Kawase & Jimbo (2018) reported that one of the proteins identified as reactive to anti-sperm antibodies from Sika wild deer is alpha-enolase (ENO1). Alpha-enolase is found in most cellular components, particularly in the cytoplasm, but can also be found on the cell surface and in the nucleus (Pancholi & Fischetti, 1998). The protein analysis results of the membrane surface of bovine sperm showed that ENO1 is localized on the membrane surface of sperm (Byrne *et al.*, 2012). According to Jiang *et al.* (2015), ENO1 is found in human sperm in the body (head/midpiece) and tail.

Alpha-enolase (ENO1) or 2-phospho-D-glycerate hydrolase with a molecular weight of 48 kDa (Liu *et al.*,

2018) is a multifunctional glycolytic enzyme expressed on the membrane surface of various cell types (Diaz-Ramos *et al.,* 2012). Each isoenzyme of alpha-enolase is a homodimer consisting of 2 alpha, 2 gamma, or 2 beta subunits. Alpha-enolase is an enzyme that plays a role in energy-generating metabolism via the glycolysis pathway (Ji *et al.,* 2016). ENO1 plays a role in the second step of adenosine triphosphate (ATP) production via the glycolysis pathway, namely as a catalyst for the dehydration of 2-phospho-D-glycerate to phosphoenol-pyruvate (Kim & Dang, 2005).

Capello *et al.* (2016) reported that inhibition of alpha-enolase affects the overall state of cellular metabolism. In addition, alpha-enolase also regulates cytoskeletal filament movement (Keller *et al.,* 2007). According to Jiang *et al.* (2015), ENO1 can be used as a biomarker to predict the motility of thawed human sperm after freezing. The assumption that ENO1 plays a role in sperm adaptation to osmosis was made by Lavanya *et al.* (2021). They found low expression of the ENO1-encoding gene in Bos taurus sperm with poor adaptability to osmosis. ENO1 regulates energy production for sperm motility (He *et al.,* 2014). An individual artificial insemination fertility rate was significantly correlated with ENO1expression (Harayama *et al.,* 2017).

The presence of alpha-enolase protein has not been detected either as an intracellular protein or as part of the plasma membrane proteins in Bali cattle sperm. This study was conducted to determine the presence of alpha-enolase in the plasma membrane of sperm and its relationship to the quality of Bali cattle sperm.

## MATERIALS AND METHODS

### Time and Place of Research

This study was conducted at Singosari Artificial Insemination Center, Malang and Reproductive Rehabilitation Unit (URR) Laboratory, Division of Reproduction and Obstetrics, Department of Veterinary Clinic, Reproduction, and Pathology, Faculty of Veterinary Medicine, IPB University from August 2020 to March 2021. The Animal Ethics Committee approved all procedures performed on animals in this study of IPB under certificate number 002/KEH/SKE/I/2021.

### Collection and Examination of Fresh Semen Characteristics

A total of 30 ejaculates (semen) used in this study were obtained from 5 Bali bulls aged 5-6 years and kept as semen sources at Singosari Artificial Insemination Center, Malang. Semen collection was performed using an artificial vagina. After collection, the semen was macroscopically examined, including volume, color, consistency, and pH. In addition, motility and motility kinematics were investigated using computer-assisted sperm analysis (CASA). Sperm viability using eosin-nigrosine differential staining, plasma membrane integrity (MI) using the HOS test method, and sperm concentration were measured using a spectrophotometer at a wavelength of 546 nm. Before measurement, the semen was diluted 100 times using a 0.9% NaCl solution (Merck, Darmstadt, Germany).

### Method for Sperm Washing

Washing aims to separate and clean the sperm from the seminal plasma. For washing, four times the initial volume of ejaculate was mixed with Phosphate Buffer Saline (PBS) solution (Dulbeccos, Sigma Life Science, USA) and then centrifuged at 500 x g for 15 minutes. After discarding the supernatant, another dilution was performed to obtain sperm with a concentration of approximately $1x10^6$ sperm/mL. The washing procedure was performed four times (Tripathi *et al.,* 1999). The processed semen is frozen at -20 °C for temporary storage and transport.

Washing is intended to ensure that sperm motility, motility kinematics, viability, and the measured plasma membrane integrity (MI) are not affected by substances in the seminal plasma. For ENO1 measurement, washing ensures that the amount of ENO1 measured is the ENO1 contained in the plasma membrane of the sperm.

### Measurement of Sperm Motility, Viability, and Plasma Membrane Integrity

Total motility, progressive motility, and motility kinematics (VCL: Curvilinear velocity, VSL: Straight-line velocity, VAP: Average path velocity, ALH: Lateral head displacement, and BCF: Beat-cross frequency) were measured using the Computer Assisted Sperm Analysis (CASA) IVOS II (The Hamilton Thorne, USA). Measurement of viability and MI of sperm was also conducted as supporting data. Viability was measured through the eosin-nigrosine differential staining method (Agarwal *et al.,* 2016). MI was calculated through the HOS-Test method (Ramu & Jeyendran, 2012).

Sperm motility, viability, and MI measurements were performed on fresh and washed semen. The procedures for measuring motility and kinematics of motility, viability, and MI are as follows:

**Motility measurement using CASA.** Before measuring total motility, progressive motility, and motility kinematics, fresh or washed semen was first diluted with a 0.9% NaCl solution with a dilution level of 100. Then, one drop or ± 10-20 μL of the diluted semen was placed on an object-glass and covered with a coverslip. Afterward, the object-glass was placed on CASA IVOS II to measure sperm motility and motility kinematics.

**Sperm viability measurement using eosin-nigrosine differential staining.** Viability is measured by placing one drop of fresh or washed semen on an object glass. The drop of semen was then mixed with two drops of eosin solution prepared from 0.5 g eosin Y in 50 mL distilled water. The semen-eosin mixture was stirred for 15 seconds until it was evenly distributed. It was then drizzled with two drops of nigrosine solution made from 5 g nigrosine in 50 mL distilled water and stirred for 15 seconds until completely mixed. One drop of the semen-eosin-nigrosine mixture or ± 10-20 μL was taken

from the object-glass. It was mixed and placed at the end of another object-glass. The semen-eosin-nigrosine mixture was then smeared onto the empty surface of the object-glass using another object-glass. The smear was dried above a spiritus lamp until the smear was dry.

The number of viable sperm was determined using a light microscope at 40x magnification. Sperm were considered viable if the sperm head was not stained with eosin-nigrosine. The number of sperm counted for viability measurement was 230 in 5 fields.

**Plasma membrane integrity measurement using HOS-Test.** MI measurement was performed by mixing 0.1 mL of fresh semen or washed semen with 1 mL of HOST solution consisting of 1.35 g fructose and 0.735 g sodium citrate in 100 mL distilled water. Then the mixture of semen and HOST solution was incubated at 37 °C for 30 minutes. After 30 minutes, one drop of the HOST-semen solution mixture was taken, placed on a glass slide, and then covered with a coverslip. The number of swollen sperm was counted using a light microscope at 40x10 magnification. A coiled tail characterizes sperm with plasma membrane integrity. The number of sperm counted was 200 from 5 fields of view.

**The measurement of eno1 amount by enzyme-linked immunosorbent assay (ELISA).** The quantity of ENO1 with the ELISA method was measured through the sample preparation stage and absorbance reading. Sample preparation and placement on the plate before absorbance measurement were performed according to the protocol included in the bovine alpha-enolase (alpha ENO) ELISA kit (MyBioSource, Sandiego, USA):

**1. Sample preparation**

The preparation procedures performed included the preparation of standard samples and semen samples and the placement of standard samples and semen samples on a wall plate. Preparation of standard samples was performed according to the following procedure: 1) the kit was removed from the refrigerator and allowed to stand for 20 minutes; 2) washing solution contained in the kit was diluted with distillate water by a ratio of 1: 25 dilutions; 3) the bovine $\alpha$ENOL standard sample was diluted with 1.0 mL of standard sample diluent to obtain a concentration of 10.0 ng/mL and allowed to stand for 30 minutes; 4) to obtain concentrations of 5.0 μL, 2.5 μL, 1.25 μL, 0.625 μL, 0.312 μL, and 0.156, 300 μL of the standard sample was re-diluted with the standard sample diluent, while only the standard diluent was used for the negative control (blank).

Sample preparation was performed by re-diluting and homogenizing the washed semen samples. Thawing was performed in a water bath at 37 °C for 15 minutes. Homogenization was performed with a shaker.

Placement on the well plate was performed according to the following procedure: 1) 100 μL of the standard and semen samples were added to the wells and sealed with the sealing cap provided in the kit, then the plate was incubated at 37 °C for 90 minutes; 2) thirty minutes before the end of the incubation, the bovine ENO1 antibody was added with a dilution of 1: 100; 3) after incubation, the plate was washed twice for 20-30 seconds; 4) after washing, 100 μL of diluted bovine ENO1 antibody was added and then incubated for 60 minutes at 37 °C; 5) 30 minutes before the end of incubation, the conjugated enzyme was diluted with a dilution of 1: 100 dilution; 6) after incubation, the plate was washed three times; 7) after washing, 100 μL of the conjugated enzyme was added and incubated at 37 °C for 30 minutes; 8) after completion of incubation, 100 μL of color reagent was added and incubated at 37 °C for 30 minutes; 9) after 30 minutes of incubation, 100 μL of color reagent C was added.

**2. Absorbance reading**

Absorbance measurements to determine the standard curve and ENO1 amount of the samples were performed using an ELISA reader (Biotek, Gen5 software, USA). Measurements were performed at a wavelength of 450 nm for 10 minutes.

### Data Analysis

The effect of motility level on the amount of alpha-enolase was analyzed using a completely randomized block design (CRBD). Linear Regression analysis, Pearson's correlation, and The Student's t-test were used to determine the relationship between motility, viability, and plasma membrane integrity of sperm and the amount of alpha-enolase.

## RESULTS

### Semen Sample Characteristics

The results from fresh semen evaluation indicate whether the bull can be used as a semen source. In this study, the semen samples had 60.60%-96.20% total motility and 57.90%-91.70% progressive motility. The average fresh semen sample quality indicators (volume, pH, concentration, total motility, and progressive motility) are presented in Table 1.

Table 1 shows that the semen sample used in this study has good quality. These results also showed that all of the male Bali cattle used in this study produced good-quality semen.

### Semen Quality and Plasma Membrane Alpha-Enolase Quantity of Sperm

The indicators of sperm quality and ENO1 quantity of sperm plasma membrane are shown in Table 2. The ENO1 target in this study is ENO1 in the plasma membrane of the sperm and not intracellular ENO1. Since the preparation procedure does not degrade the sperm, only ENO1 can be detected in the plasma membrane. It can assure that ENO1 from the measurements performed in this study is ENO1 contained in the plasma membrane and not derived from seminal plasma. Before measurement, sperm are repeatedly washed with a protein-free washing solution to free them from other substances,

especially substances in the form of proteins contained in seminal plasma.

The average amount of ENO1 plasma membrane sperm from the Bali cattle in the study was 1.27 ng/$10^6$ sperm. The analysis of variance showed that the amount of ENO1 plasma membrane sperm from the ejaculations of the five bulls was not significantly different (Table 4). In addition, the amount of ENO1 in the sperm plasma membrane increases with sperm concentration when ENO1 is bound or is an integral part of the sperm plasma membrane. The amount of ENO1 in the plasma membrane of sperm increases significantly with the increasing sperm concentration ($p<0.01$; Table 3).

Motility, viability, and plasma membrane integrity are indicators of semen quality that can determine sperm fertility. Total sperm motility, progressive sperm motility, viability, and plasma membrane integrity are shown in Table 2. The regression analysis results, Pearson's correlation, and The Student's t-test showed no relationship between total motility, progressive motility, viability, and plasma membrane integrity with the amount of ENO1. The correlation coefficient (r) of the relationship between total motility, progressive motility, viability, and plasma membrane integrity and the amount of ENO1 was shown in Table 2.

Table 1. Semen sample characteristics

| Variables | Range | Mean±SD |
|---|---|---|
| Semen volume (mL) | 1.50-10.00 | 5.3±2.03 |
| pH | 6.2-6.8 | 6.5±0.20 |
| Sperm concentration ($10^6$/mL) | 466-1360 | 926.87±247.83 |
| Sperm motility (%) | | |
| TM | 66.80-96.90 | 80.97±8.82 |
| PM | 52.80-90.60 | 72.50±9.40 |
| Kinematics of sperm motility | | |
| VCL (μm/s) | 114.64-263.59 | 188.64±19.44 |
| VSL (μm/s) | 58.94-177.77 | 111.19±10.15 |
| VAP (μm/s) | 72.36-181.53 | 120.78±12.26 |
| ALH (μm) | 4.07-9.23 | 62.9±0.83 |
| BCF(Hz) | 15.31-39.09 | 30.07±4.03 |
| Viability (%) | 68.26-97.83 | 82.66±8.78 |
| MI (%) | 69.13-98.26 | 85.05±8.15 |
| ENO1 amount (ng/$10^6$ sperm) | 0.97-1.89 | 1.27±0.26 |

Note: VCL= Curvilinear velocity, VSL= Straight-line velocity, VAP= Average path velocity, ALH= Lateral head displacement, BCF= Beat-cross frequency, TM= Total motility, PM= Progressive motility, MI= Membrane integrity.

The role of ENO1 is one of the catalysts in the process of energy formation for sperm survival and motility. The amount of ENO1 in the plasma membrane of sperm at three motility levels is shown in Table 4. The amount of ENO1 is relatively the same at the three motility levels. The analysis of the variance of the amount of ENO1 in the three motility levels showed no difference in the amount of ENO1 in the plasma membrane of sperm in the three motility levels (Table 4).

### Relationship Between Motility Kinematics and Alpha-Enolase Amount

Motility kinematics is a parameter generated by the CASA. Motility kinematics parameters describe sperm motility characteristics. The main parameters of motility kinematics and ENO1 amount of Bali cattle sperm from the study are shown in Table 2.

The results of regression analysis and Student's t-test showed that there was no correlation between the parameters of motility kinematics (VCL, VSL, VAP, ALH, and BCF) and the amount of ENO1 in the plasma membrane of sperm. The correlation coefficients (r) between VCL, VSL, VAP, ALH, and BCF and the amount of ENO1 are shown in Table 2.

## DISCUSSION

All semen sources used in this study had good sperm quality (Table 1). According to Prastowo *et al*. (2018), the semen volume of Bali bulls ranged from 4.55-5.18 mL, pH was 6.51-6.52, sperm concentration was 962.30-1079 x $10^6$/mL, and sperm motility was 66.4%-58%. The data obtained (Table 1) demonstrate

Table 2. Pearson correlation and linear regression between alpha enolase (ENO1) (ng/$10^6$ sperm) to the quality of Bali cattle sperm

| Variables | Equation | R | $R^2$ | p-value |
|---|---|---|---|---|
| Semen volume (mL) | Y= 5.290+0.005X | 0.001 | 0.000 | 0.997 |
| pH | Y= 0.987+0.044X | 0.028 | 0.001 | 0.884 |
| Sperm concentration ($10^6$/mL) | Y = 1036.421-86.128X | -0.092 | 0.008 | 0.629 |
| Sperm motility | | | | |
| TM | Y= 0.771+0.006X | 0.206 | 0.043 | 0.274 |
| PM | Y= 1.017+0.04X | 0.125 | 0.016 | 0.511 |
| Kinematics of sperm motility | | | | |
| VCL (μm/s) | Y= 186.402+1.760X | 0.013 | 0.000 | 0.946 |
| VSL (μm/s) | Y = 1.122+0.001X | 0.145 | 0.021 | 0.445 |
| VAP (μm/s) | Y = 1.146+0.001X | 0.106 | 0.011 | 0.577 |
| ALH (μm) | Y = 1.545-0.043X | 0.193 | 0.037 | 0.306 |
| BCF(Hz) | Y = 0.903+0.012X | 0.286 | 0.082 | 0.126 |
| Viability (%) | Y = 0.811+0.006X | 0.185 | 0.034 | 0.328 |
| MI (%) | Y= 0.854+0.005X | 0.151 | 0.023 | 0.426 |

Note: VCL= Curvilinear velocity, VSL= Straight-line velocity, VAP= Average path velocity, ALH= Lateral head displacement, BCF= Beat-cross frequency, TM= Total motility, PM= Progressive motility, MI= Membrane integrity, R= Pearson's correlation coefficient, $R^2$=Determination coefficient.

Table 3. Alpha enolase (ENO1) quantity at five washed sperm concentration levels

| Sperm concentration ($10^6$/mL) | ENO1 quantity (ng) | |
|---|---|---|
| | Range | Mean ± SD |
| $6.25x10^4$ | 0.11-0.15 | $0.13±0.02^A$ |
| $1.25x10^5$ | 0.23-0.34 | $0.28±0.05^B$ |
| $2.50x10^5$ | 0.45-0.53 | $0.49±0.03^C$ |
| $5.00x10^5$ | 0.81-1.04 | $0.88±0.11^D$ |
| $1.00x10^6$ | 1.28-2.16 | $1.65±0.34^E$ |

Note: Means in the same column with different capital letter superscripts differ highly significantly ($p<0.01$).

that ENO1 is one of the components of the plasma membrane of Bali cattle sperm. Likely, ENO1 can also determine the fertility of Bali cattle sperm. Byrne *et al.* (2012) reported that using mass spectroscopy, one of the proteins identified on the surface of the plasma membrane of Brahman cattle sperm is ENO1. ENO1 is a glycolytic enzyme located in the cell cytoplasm (Diaz-Ramos *et al.*, 2012).

Some studies have shown that ENO1 is also found in the other parts of the sperm (Petit *et al.*, 2013). Kasvandik *et al*. (2015) reported that ENO1 is one of the proteins found in the anterior and posterior heads of sperm in mice, pigs, and humans. ENO1 is also found in the tail of human sperm (Rahman *et al.*, 2013) and in mice (Gitlits *et al.*, 2000). Recently, ENO1 was reported to be one of the proteins found in the seminal plasma of Angus crossbred cattle (Zoca *et al.*, 2022). Enolase is an enzyme that acts as a catalyst for the hydrolysis of 2-phospho-D-glycerate to phosphoenolpyruvate in the final phase of glucose catabolism via glycolysis pathway and as phosphoenolpyruvate hydratase in the gluconeogenesis pathway (Diaz-Ramos *et al.*, 2012). The average amount of ENO1 in the plasma membrane of sperm determined in this study was 1.27 ng/$10^6$ sperm (Table 1). In the human sperm group with a high physiological level, ENO1 is one of the under-expressed sperm proteomes (Agarwal *et al.*, 2016a). Park *et al*. (2022) reported that low-motility bovine spermatozoa expressed high ENO1 in the head region.

These results show that the plasma membrane of Bali cattle sperm contains ENO1. Sperm quality was not related to the amount of ENO1 (Table 2). The presence of ENO1 in the plasma membrane did not affect total and progressive motility. This fact suggests that ENO1 present in the plasma membrane does not play a direct role in providing energy in the form of ATP via the glycolysis pathway to the sperm and thus has no effect on sperm motility or motility levels. ENO1 in the plasma membrane of sperm is not a fertility-inhibiting substance and is not toxic to sperm. This fact is evidenced by the lack of a relationship between the amount of ENO1 in the plasma membrane of sperm and the viability and integrity of the plasma membrane of sperm. Mortimer *et al*. (2013) found chemical residues, endotoxins, and other biological toxins affect sperm survival. Glycolysis in sperm occurs in the main part of the flagellum, and several glycolytic enzymes have been found to reside in the flagellar envelope (Turner, 2006).

Table 4. Alpha-enolase (ENO1) amount at three total motility levels of washed sperm

| Motility levels (%) | ENO1 amount (ng/$10^6$ sperm) |
|---|---|
| < 70 | 1.01±0.18 |
| 70 - 79 | 1.14±0.20 |
| > 80 | 1.06±0.15 |

The facts presented in Table 4 show no correlation between the amount of ENO1 in the plasma membrane of sperm and the degree of motility. This result might be due to glycolysis is not the main factor in providing energy for sperm motility. Most ATP molecules required for sperm motility come from the respiratory pathway (oxidative phosphorylation). According to du Plessis *et al.* (2014), ATP production via respiration is more efficient than glycolysis. It has been shown that inhibition of glycolysis does not affect human sperm motility. This fact is not in line with the research results of Park *et al*. (2022), who reported that a group of low-motility human sperm expressed high ENO1 in the head region. It is further hypothesized that high ENO1 will cause an increase in the utilization of energy sources for sperm, thereby accelerating the depletion of energy sources for motility, resulting in low motility.

Sperm viability and motility can still be maintained because ENO1 plays a role in sperm plasma membrane stability. Lee *et al*. (2017) stated that the protein contained in the plasma membrane of sperm is directly related to the viability and motility as well as the level of integrity of the plasma membrane of sperm. According to Didiasova *et al*. (2019), one of the essential roles of ENO1 in cells is to maintain the stability of the mitochondrial plasma membrane. Based on the results of the proteomic analysis in normozoospermia, ENO1 is one of the proteins associated with sperm motility, viability, and acrosomal plasma membrane integrity (Hezavehei *et al.*, 2018). Damage to the plasma membrane negatively correlates with the Nelor bovine sperm motility (Reis *et al.*, 2016).

The amount of ENO1 in the plasma membrane of sperm increases with increasing sperm concentration (Table 3). This fact shows that the plasma membrane of Bali cattle sperm contains ENO1, which depends on the concentration of sperm. This condition is normal because ENO1 is an integral protein part of the sperm's plasma membrane.

Motility is the most important sperm quality parameter that will determine the success of fertilization, both naturally and artificially (Dcunha *et al.*, 2022). CASA-generated kinematic motility describes sperm head motion along the trajectory traveled during sperm movement (Gallagher *et al.*, 2019). The eight parameters generated by CASA include sperm motion velocity, velocity ratio, and wobble properties. According to Soler *et al*. (2017), the main parameters measured with CASA are VCL, VSL, VAP, ALH, and BCF, while LIN, STR, and WOB are ratio measures of the main parameters. The sperm motility velocity characteristics determined by CASA are helpful for the assessment of sperm fertility (Nagy *et al.*, 2015). Kinematics motility correlates with

the overall motility and progressive motility of bovine sperm (Pathak *et al.,* 2019).

There is no correlation between the parameters of motility kinematics and the amount of ENO1, because the presence of ENO1 does not affect sperm motility and thus does not alter the sperm movement pattern. The lack ENO1 effect on motility is since the energy required for viability and motility is sufficient even without the contribution of ENO1 in the plasma membrane of sperm but with the help of oxidative phosphorylation. This condition is evidenced by the high viability and plasma membrane integrity (Table 2). Kwon *et al.* (2012) reported that VCL, VAP, and ALH were positively correlated with porcine sperm motility.

The average values of VCL, VSL, VAP, ALH, and BCF of Bali cattle sperm obtained from this study (Table 4) were included in the fast motility criteria due to the washing process and higher than those reported by Yulnawati *et al.* (2014) namely VCL= 184.5 μm/s; VSL= 61.5 μm/s; VAP= 90.0 μm/s; ALH=6.6 μm, and BCF=22.6 Hz. It was further reported that single layer centrifugation could improve the motility kinematics of cattle sperm.

## CONCLUSION

This study concluded that alpha-enolase (ENO1) can be detected on the plasma membrane of Bali cattle sperm but does not determine sperm quality and an antifertility substance.

## CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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