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Sperm osteopontin mRNA expression levels and its correlation on semen quality and fertility in Madura bulls

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Abstract. *Rosyada ZNA, Yoelinda VT, Kaiin EM, Gunawan M, Ulum MF, Tumbelaka LITA, Solihin DD, Agil M, Gunawan A, Purwantara B. 2023. Sperm osteopontin mRNA expression levels and its correlation on semen quality and fertility in Madura bulls. Biodiversitas 24: 563-570.* Osteopontin (OPN) gene transcripts influence spermatogenesis and germ cell development. Therefore, transcriptome analysis is needed to identify the fertility factor OPN in Madura bull sperm. Madura cattle are crosses originating from *Bos indicus* (zebu) and *Bos javanicus* (banteng). This study aims to examine sperm osteopontin (OPN) mRNA expression levels and its correlation with semen quality and fertility in Madura bulls. Frozen semen samples from Madura bulls were categorized as high-fertile (n: 4, average field conception rate: 78.28±3.25%) or sub-fertile (n: 4, average field conception rate: 66.73±5.01%). In post-thaw semen samples, sperm motility, viability, membrane, acrosome, and DNA fragmentation index were evaluated. OPN expression in sperm total RNA was analyzed using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). The data analysis between the two fertility groups was assessed with Student's t-test and Pearson Square. Receiver-operating characteristic analysis and diagnostic sensitivity and specificity calculations were performed. Sperm motility, acrosome intact, and DFI differ ($p<0.05$) between groups, whereas viability and membrane plasma intact have no significance ($p>0.05$). In high-fertile Madura bulls, OPN mRNA was upregulated ($p<0.05$). The OPN mRNA expression had a strong correlation with the field conception rate (R: 0.807, $P<0.05$), sperm motility, and intact acrosome (R: 0.899, $p<0.01$; R: 0.804, $p<0.05$) respectively. In contrast, OPN negatively correlated with sperm DFI (R: -0.764, $p<0.05$). The OPN predicted bull fertility with 81.3% accuracy, 75% sensitivity, and 75% specificity. Thus, OPN may serve as a potential biomarker of Madura bull fertility.

Keywords: Biomarker, RT-qPCR, sperm evaluation, transcriptome

INTRODUCTION

Madura bulls are being bred to fulfill the need for meat in Indonesia as one of the country's indigenous livestock and germplasm. Madura bulls are the result of a crossbreeding of the *Bos indicus* (*zebu*) and *Bos javanicus* (*banteng*). Madura bulls are more adaptable to local conditions than other breeds, can grow well even with low diet quality, and have a high carcass with good carcass quality (Sutarno and Setyawan 2016; Kutsiyah 2017). Unfortunately, Madura bulls widely developed by small farmers in Madura Island are known to have poor reproductive performance, so their population is decreasing (Zuhri et al. 2019). Artificial insemination with frozen sperm can be used to increase the population of native Indonesian cattle (Zelpina et al. 2012). The bulls that have been used in national and regional insemination centers in Indonesia are superior. The superior Madura bulls are bulls that have been selected based on seed quality standards (Regulation No. 10/Permentan PK.210/3/2016 of the Minister of Agriculture of the Republic of Indonesia). Superior bulls according to Indonesian National Standard (SNI) of frozen semen number 4869 of 2017 part 1 are normal bulls that have been selected based on their ancestry (pedigree/pedigree), production, and reproductive capacities, as well as being healthy and free of infectious diseases. Therefore, the chosen bull for the seed superior bulls has no inbreeding problems. In addition, there is also an AI record for cattle from the inseminator that performed the AI in the field to prevent inbreeding. Thus, artificial insemination will be successful if using high-quality frozen semen, which has passed both microscopic and macroscopic evaluations and has been combined with transcriptomic analysis.

Studies using omics techniques, such as transcriptome and proteomic analysis of bovine seminal plasma and sperm over the past few decades have considerably enhanced our understanding of the molecular pathways

involved in bull fertility and semen preservation (Moura and Memili 2016; Ugur et al. 2019; Pardede et al. 2020). Based on these studies, several proteins were proposed as candidates for fertility markers (Selvaraju et al. 2017; Pardede et al. 2020; Hitit et al. 2021) and sperm cryo-resistance (Rego et al. 2016; Moura et al. 2018). Cancel et al. (1999) discovered fertility-associated proteins in Holstein's seminal plasma and sperm. Osteopontin was linked to reproductive characteristics (Cancel et al. 1999; Erikson et al. 2007; Preedaa et al. 2020; Filho et al. 2021).

Osteopontin (OPN) plays a role in fertilization, bone remodeling, immunological modulation, inflammation, and vascularization (Singh et al. 2018; Vianello et al. 2020; Willforss et al. 2021). Supplementation of OPN in *in vitro* medium or sperm treated with OPN resulted in higher fertilization, cleavage, and embryo development in cattle (Moura et al. 2018), *Bubalus bubalis* (buffalo) (Boccia et al. 2013), and *Sus scrofa domesticus* (porcine) (Chen et al. 2022). These findings provide credence to the idea that OPN is a protein that is required for successful fertilization and early embryo development.

In addition to the roles that OPN plays in the oviduct during fertilization, there is strong evidence to suggest that it also plays a role in the physiology of sperm. OPN is secreted in the male reproductive organs, and it is present in the seminal plasma, where it has the potential to bind to sperm. Interestingly, OPN was shown to be significantly abundant in the seminal plasma of bulls that presented high semen freezability (Rego et al. 2016). Nonetheless, it is yet unknown how OPN expresses in sperm or how it influences the functionality of sperm. Furthermore, there is still a lack of evidence concerning the impact of OPN mRNA expression in sperm, particularly about structural and functional parameters like motility, viability, acrosome and membrane functions, and DNA integrity. Despite this, the increased sperm capacitation and acrosome reaction found in bull (Monaco et al. 2009; Willforss et al. 2021) and buffalo (Boccia et al. 2013) sperm imply that OPN directly alters sperm functional parameters.

The fact that OPN is a candidate for reproductive biomarkers in the bull is supported by all the aforementioned studies or research. On the other hand, there is still a lack of information on this gene and its possible role as a molecular marker, such as whether or not OPN is associated with the quality of bull sperm. The enhancement of bull reproductive efficiency would be made possible with the utilization of OPN as a biomarker. Therefore, the objective of this study was to analyze the levels of OPN mRNA expression in the sperm of Madura bulls and establish if there was a correlation between these levels and the sperm quality and fertility of the bulls.

MATERIALS AND METHODS

Ethical statement

This study was excluded from the Animal Care and Use Committee's ethical clearance evaluation since artificial vagina semen collection doesn't alter animal physiology. This study followed SNI ISO 9001: 2015 No. 824 100 16072 at the Lembang AI center and SNI ISO 9001: 2015 No. G.01-ID0139-VIII-2019 at the Singosari AI center, supervised by a veterinarian from both institutions. Lembang and Singosari AI center's ethical committee offered ethical guidance and clearance for the responsible usage of bulls for semen collection. In addition, semen collection using an artificial vagina is performed by an experienced bull technician.

Frozen semen sample collection

Samples of frozen semen from Madura bulls were obtained from the Semen Bank at the National AI Center in Lembang, Bandung, West Java, and Singosari, Malang, East Java, Indonesia. Semen samples from Lembang AI Center were processed according to the AI center's standard operating procedure (SOP) which used skim milk as the extender. The composition of the skimmed milk diluent in 1000cc was 100g of skimmed milk, 960cc of distilled water, and antibiotic (3000000 IU of penicillin, 3g of streptomycin, and 30cc of distilled water). The ratio between skimmed milk diluent and antibiotic was 100:1. Semen samples from Singosari, on the other hand, were cryopreserved using tris egg yolk. The composition of the tris egg yolk extender was 20% of egg yolk, 1.6% of tris aminomethane, 1.4% of lactose, 2.5% of raffinose, 0.9% of citric acid, and antibiotic (1000000 IU/L of penicillin, 1g/L of streptomycin, and 1g/L of distilled water). Lembang and Singosari AI centers respectively use extenders in the form of skim milk and tris egg yolk supplemented with antibiotics. The extender in each artificial insemination center functions as a cryoprotectant that protects sperm during freezing increases semen volume, and maintains sperm viability and sperm pH; thus, the frozen semen production process requires an extender to maintain sperm quality. The frozen samples of sperm were transported using a transport container containing -196°C liquid nitrogen. The samples were stored in the container until further analysis.

Eight productive Madura bulls (4-8 years old) were used for this study. Based on the fertility rate (%) from about 1500 inseminations per bull, they were put into two groups: high-fertile (n: 4) and sub-fertile (n: 4). Classification of bull fertility levels was carried out using data from the East Java Province national animal health information system (iSIKHNAS) for 2018-2020. The reproductive efficiency data is in the form of AI data and pregnancy examination. The data used are AI data with the number of acceptors >100 cows that were successfully pregnant at the first AI using the same bull ID. Bull fertility is then grouped based on the results of the percentage of conception rate (%CR) calculation which is tabulated in the form of mean and standard deviation (sd) according to the method (Aslam et al. 2018; Singh et al. 2018). Bulls with %CR values less than or below "mean - 1Sd" are classified into the sub-fertile, while bulls with %CR more than or above "mean + 1Sd" is included in the high fertile. The mean fertility rate for high-fertile bulls was 78.28±3.25%, while it was 66.73±5.01% for sub-fertile bulls. Since many inseminations were done to find field conception rates, environmental, management, and cow effects on bull

fertility were thought to be minimal (Aslam et al. 2018). Three different batches and five replication of frozen sperm samples respectively were tested for their functional sperm parameters.

Sperm characteristic parameters

A total of 15 straws from three different batches in each bull were evaluated in this study. The frozen semen samples were thawed in a water bath at 37°C for 30 s and then subjected to sperm characteristic evaluations. Microscopically, frozen semen samples were analyzed from several parameters including motility, viability, integrity of the plasma membrane, and acrosome, and sperm DNA fragmentation index. Sperm motility parameter analysis was performed using computer-assisted sperm analysis (CASA) (Minitüb, Tiefenbach, Germany) with the sperm vision 3.7 programs. To assess total motility, progressive motility refers to the method of Sundararaman et al. (2012). In brief, sperm motility was evaluated using CASA, Spermvision™ 3.7 (Minitub, Germany). CASA was connected to an Olympus BX 51 microscope (Olympus, Tokyo, Japan) and a 37°C heating table. Preparation and observation of sperm motility were carried out according to the manual book from Spermvision™ 3.7 (Minitub, Germany). Frozen semen was thawed at 37°C for the 30s and then put into a centrifuge tube. 10 µL of semen was diluted 10 times using physiological saline. As much as 10 µL of diluted semen was then dripped onto a glass object and covered with a warm glass cover. Sperm motility was observed using a 20x magnification objective lens with five fields of view and the number of sperm cells ranged from 50-250 in each field of view. Sperm viability was analyzed using eosin nigrosine staining. The live sperm were clear (transparent), whereas the dead sperm took on red (Figure 1) (Björndahl et al. 2003). Observations were made with a 400X magnification microscope in 10 fields of view and the number of cells was 200 cells. Sperm plasma membrane integrity parameters were analyzed using fluorescence staining with Carboxyfluorescein diacetate-propidium iodide (CFDA-PI; Sigma Aldrich, Germany) was used to analyze plasma membrane integrity. Sperm with intact membranes fluoresced brilliant green, but damaged sperm membranes fluoresced bright red (Figure 1) (Harrison and Vickers 1990). Sperm acrosome cap analysis using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; Sigma Aldrich, Germany) Sperm with an undamaged acrosome displayed a strong green acrosomal cap fluorescence. While spermatozoa with a damaged acrosome had a red fluorescence cap (Figure 1) (Cheng et al. 1996). Analysis of sperm DNA fragmentation index (DFI) assessed using acridine orange (AO) staining. Green fluorescence is observed in sperm that have a normal DNA content. In contrast, the fluorescence of sperm with a defective DNA content ranges from yellow-green to reddish-orange (Figure 1) (Said et al. 2003). The sperm samples were examined under a fluorescent microscope (AxioPhot Zeiss; 490/530 nm excitation filter) using a filter at 400X magnification.

Isolation of total RNA and cDNA synthesis

Direct-zol RNA Miniprep Plus was used to separate extract RNA from spermatozoal RNA (Zymo Research, US). The sperm RNA solution contained in the RNase-free tube was stored at -80°C until further analysis. A spectrophotometer (NanoDrop, ND-1000, Thermo Scientific, USA) was used to assess RNA quality and quantity. RNA samples with a ratio of 260/280 1.8 to 2.0 were considered for cDNA synthesis. The cDNA was synthesized using the SensiFast cDNA synthesis kit (Meridian Bioline, USA), according to the manufacturer's protocol. The cDNA synthesis procedure was carried out by adding several reactions with a total volume of 20µL. The total volume of RNA used was 10µL. The protocol used for cDNA synthesis refers to the manufacturer's protocol using the MiniAmp Thermal Cycler (Applied biosystem, Thermoscientific, USA) as the primary annealing step at 25°C for 10 min, reverse transcription at 42°C for 15 min, and activation of reverse transcriptase at 85°C for 5 min, then cooled at 4°C. Then the cDNA samples were stored at -20°C until used for real-time quantification polymerase chain reaction (RT-qPCR) analysis.

Figure 1. Sperm characteristic evaluation. A Sperm viability evaluation using Eosin-Nigrosin; B Sperm membrane integrity using CFDA-PI fluorescence staining; C FITC-PNA fluorescence staining to evaluate sperm acrosome integrity; D AO fluorescence staining for evaluating sperm DNA fragmentation index

Real-time quantification PCR (RT-qPCR) analysis

RT-qPCR analysis was performed using the Sensifast SYBR mix (Thermo scientific-USA) and 2µL of cDNA per reaction in real-time PCR (CFX Opus 96, Biorad). The cycle conditions used were initial denaturation of 95°C for 2 min; 40 cycles of 95°C for 10 s and annealing temperature according to a primer of each gene (Table 1) for 30 s. Each process is duplicated and includes a non-template control (NTC). The resulting expression values were standardized to PPIA, the 'housekeeping gene'. RT-qPCR expression data for each gene were quantified using the $2^{-\Delta\Delta CT}$ technique (Livak and Schmittgen 2001).

Statistical analysis

The Madura bull sperm characteristics between the two fertility groups were assessed using Student's t-test. The data were normally distributed based on the Kolmogorov-Smirnov test. Quantitative data were expressed as the standard deviation of the mean. Data were tested for normal distribution using the Kolmogorov-Smirnov test. Pearson correlation was used to examine the relationship between sperm characteristics, OPN expression, and fertility groups. The receiver operating Characteristic (ROC) curve was calculated with the area under curve (AUC) values in SPSS. AUC values ≥0.7 were considered to yield good diagnostic accuracy (Yang and Berdine 2017). Data analysis was performed using SPSS statistical software (IBM, New York, NY, United States).

RESULTS AND DISCUSSION

Sperm functional parameters

The Madura bulls were divided into two categories: high-fertile (n: 4) and sub-fertile (n: 4) according to the average conception rate of the bulls. In addition, the study of the post-thawed sperm showed that there were significant differences ($p<0.05$) between high-fertile and sub-fertile in total sperm motility (53.87±1.74a) and (50.85±0.45b), in acrosome intact (83.17±4.10b) and (72.14±6.73b), and in sperm DFI (14.58±1.31a) and (17.27±0.89b). On the other hand, there was not a significant difference ($p>0.05$) between high fertile and subfertile in sperm viability (82.67±1.49) and (72.50±7.66); intact membrane plasma (79.65±0.50) and (69.66±6.70) (Figure 1).

Relative mRNA expression

It was discovered that there was a significant difference ($p<0.05$) between high fertile and subfertile levels of OPN expression (0.094±0.032a) and (0.016±0.007b) (Figure 2a). When compared to sub-fertile bulls, the high-fertile bulls had higher levels of OPN expression, indicating that it was up-regulated. The ROC curve model (Figure 2b) was also carried out to determine the efficiency of OPN mRNA expression, which could, later on, be potentially used as potential markers of Madura bulls' fertility. This was done in addition to the correlation analysis between OPN mRNA and the fertility rate of Madura bulls.

Correlation of mRNA expression levels of OPN with the sperm characteristic and fertility levels of Madura bulls

OPN mRNA expression level had a strong significant positive correlation with total motility (r: 0.807, $p<0.05$), progressive motility (r: 0.899, $p<0.01$), intact acrosome (r: 0.804, $p<0.05$) and negatively correlated with sperm DFI (r: -0.764, $p<0.05$). Furthermore, OPN mRNA expression level (r: 0.887) had a strong significant correlation ($p<0.01$) with fertility rate (Table 2).

Table 1. Primer details used for PCR amplification and RT-qPCR

Genes	Primer sequence	Product size	Annealing temperature	Accession number	Reference
OPN	F: ATGCATGACGCACCTAAGAAG R: TCAATTGACCTCAGAAGAGGC	267 bp	54.5°C	AY878328	(Preedaa et al. 2020)
PPIA	F: ATGCTGGCCCCAACACAA R: CCCTCTTTCACCTTGCCAAA	100 bp	55°C	XM_001252921.1	(Ganguly et al. 2013)

Table 2. Correlation of OPN genes expression with semen attributes and fertility levels

X/Y		Total Motility	Progressive Motility	Viability	Membrane plasma intact	Acrosome intact	Sperm DFI	Fertility levels
mRNA	OPN	0.807*	0.899**	0.673	0.690	0.804*	-0.764*	0.887**

Sperm DFI, Sperm DNA Fragmentation Index; n: 8 Madura bulls; r table: 0.707; * ($p<0.05$); ** ($p<0.01$)

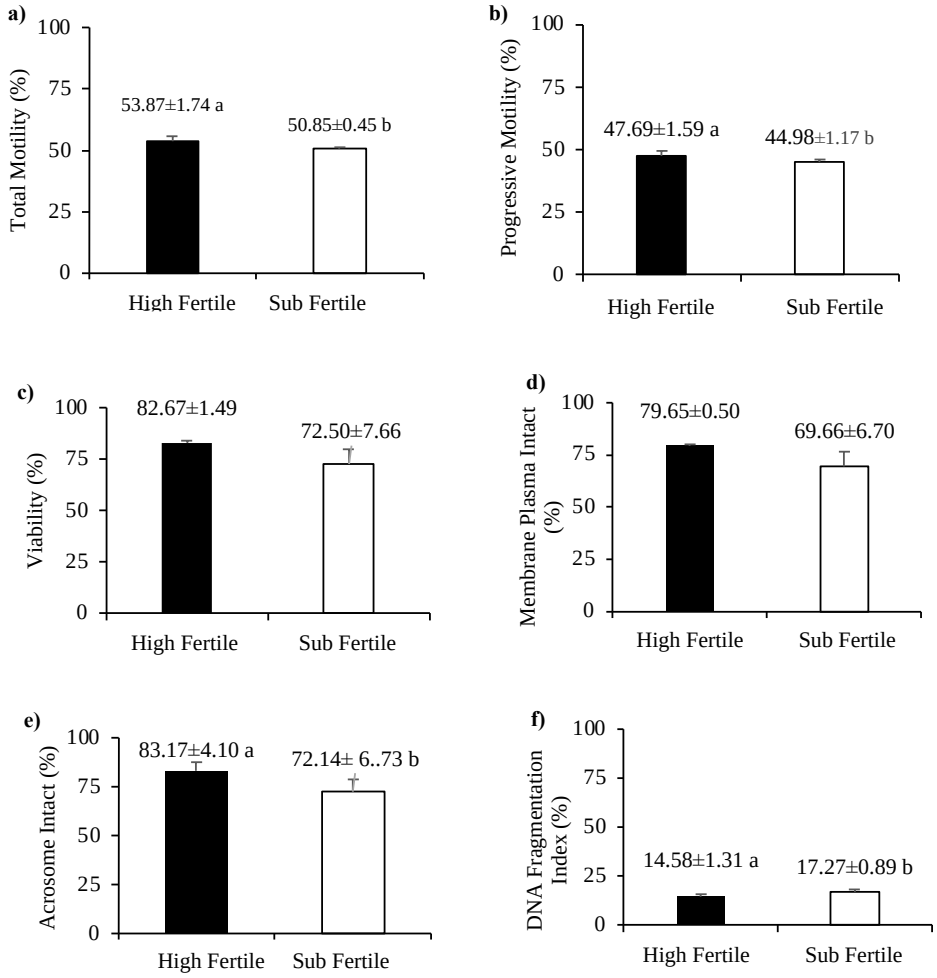

Figure 1. Sperm characteristics from Madura bulls with different fertility levels: high fertile and subfertile. a) sperm total motility; b) sperm progressive motility; c) sperm viability; d) sperm membrane plasma intact; e) sperm acrosome intact; f) sperm DFI. Data presented as mean ± SD. Significance is denoted in different superscripts (a-b) ($p<0.05$); the error bar represents the standard deviation

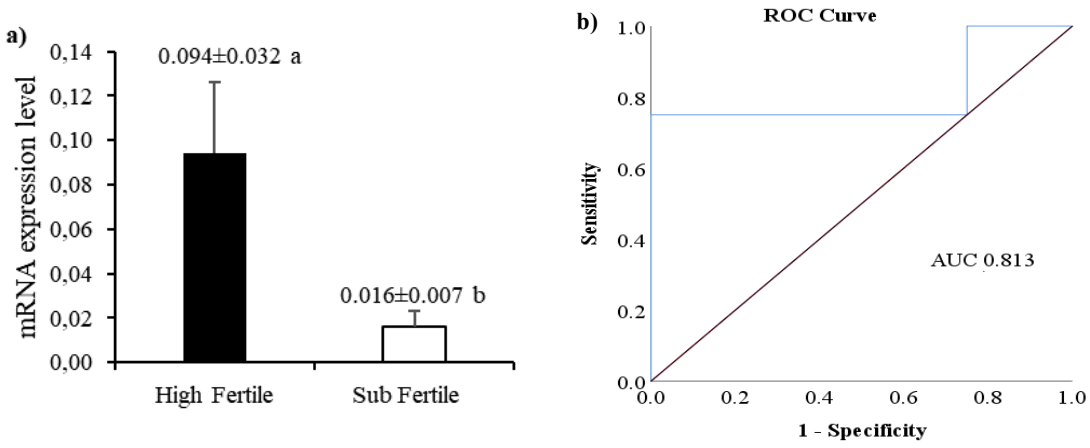

Figure 2. Regression model for predicting fertility levels in Madura bulls. a) mRNA expression level of OPN was 0.094 in the high fertile higher than sub-fertile bulls; b) ROC curve generated by logistic regression model of OPN and fertility levels. OPN expression levels had a sensitivity of 0.75 (75%) and specificity of 0.75 (75%) for predicting fertility rate. AUC is 0.813 (81.3%). The data reveals OPN expression could predict Madura bull fertility

Discussion

Over 13,000 genes have been described, and numerous biomarkers for reproductive features have been suggested (Somashekar et al. 2017; Selvaraju et al. 2018). However, putative markers like OPN still need more testing before they can be used to create new ways to evaluate bulls and analyze their sperm. In this study, the quality of the sperm and the fertility of Madura bulls were linked to how much OPN mRNA was expressed in the sperm at different levels of fertility. This study demonstrates that OPN is present in larger concentrations in the sperm of highly fertile bulls and has a strong positive association with fertility levels and other sperm characteristic characteristics. This is the first article to our knowledge that analyzes the association between sperm OPN and semen quality and fertility levels in Madura bulls. In the post-thaw semen study, significant variations between the two fertility levels were found in sperm motility, acrosome intact, and sperm DFI; however, in sperm viability and intact membrane plasma, there were no significant differences (Figure 1). In addition, the expression levels of the OPN had a significant strong positive correlation with sperm progressive motility (r: 0.899, $p<0.01$) and the fertility levels (r: 0.887, $p<0.01$); and a positive correlation with sperm total motility (r: 0.807, $p<0.05$), and acrosome intact (r: 0.804, $p<0.05$) while having a negative correlation with sperm DFI (r: -0.764, $p<0.05$). (Table 2). The motility of sperm is disrupted during cryopreservation, in addition to affecting the sperm head. Oxidative stress is imposed on sperm during the cryopreservation process, which results in cryodamage and dramatically lowers progressive motility in mammals (Rarani et al. 2019). According to a previous study that the relationship between OPN expression and sperm integrin and CD44 receptor binding is demonstrated (Souza et al. 2009; Gomes et al. 2020) and it was shown to increase sperm motility, promoting acrosomal reactions, fertilization, and early embryonic development in cattle (Monaco et al. 2009). Further, the activation mechanism of integrin and CD44 membrane receptors and signal transduction mechanisms, such as the activation of different pathways, especially Map kinase and phosphoinositide (PI) 3-kinase, which help sperm motile and produce energy (Abedin et al. 2021). As a result, a higher OPN is associated with a larger percentage of motile sperm, as was seen in this study in extremely fertile Madura bulls. Additionally, integrins-binding OPNs such as CCDC174 and PPP1R35 can influence the maturation and motility of epididymal sperm via OPN that interact with testes/sperm-enriched protein phosphatases (Goswami et al. 2019). For example, blocking the OPN-binding CCDC87 receptor in mice decreased early motility and the acrosomal reaction caused by progesterone (Wang et al. 2018). In addition, cryopreservation decreases the rate of sperm metabolism and mitochondrial activity and affects the structure of sperm chromatin, all of which have an effect on the greater level of sperm DFI (Gomes et al. 2020).

Similar to other plasma membranes, the sperm plasma membrane is made of proteins and phospholipids (Salmah et al. 2021). Typically, cells employ particular strategies to prevent uncontrolled membrane damage (Manehat et al. 2021). The cell membrane plays a crucial function in preserving the physical organelles of the cell and regulating the flow of all substrates and electrolytes required for the cell's metabolic operations. Using eosin dye, this study discriminated between viable and nonviable spermatozoa which are the dead sperm cell-stained red. The integrity of the sperm plasma membrane was then determined by CFDA-PI staining, in which sperm with intact membranes fluoresced brilliant green and sperm with damaged membranes fluoresced brilliant red (Harrison and Vickers 1990). Since fertilization requires an undamaged plasma membrane, the reproductive potential of the semen sample can be estimated based on the ratio of life to dead sperm. Sperm vitality is a biological indicator of sperm quality because more viable sperm are needed to safely reach and fertilize oocytes (Yoon et al. 2022). The results of this study showed that fresh sperm from highly fertile Madura bulls had the highest viability compared to those that were sub-fertile, although not significantly different. However, comparing to the sperm viability of the Brangus-Simmental crosses ranging from 86.00% to 87.50% (Kaka et al. 2015), whereas the sperm viability of Mithun bulls was 54.96% (Perumal et al. 2016), the results of the evaluation on the viability and integrity of the plasma membrane in Madura bulls were still in the normal range and higher than Mithun bulls. Also, the Indonesian national standard (SNI) for frozen semen production suggests that sperm motility after thawing, which is around 40%, is the most common criterion for determining good semen for AI (Badan Standarisasi Nasional 2017). Thus, this study shows that OPN can be used as a possible indicator of sperm quality and improve the accuracy of breeding soundness examinations that meet SNI frozen semen criteria.

In addition to this, it was discovered that the relative mRNA expression levels of OPN were greater in high-fertile bulls as opposed to sub-fertile bulls (Figure 2a). According to the ROC curve analysis, our findings also revealed an association of 0.813 AUC values between the levels of OPN mRNA expression and the fertility levels of the subjects, which suggests that OPN may have a good capacity for diagnostic accuracy (Figure 2b). According to a prior study AUC values$\geq$0.7 were deemed to have adequate diagnostic accuracy (Yang and Berdine 2017). The genes that are expressed differently may play a role in spermatogenesis and affect how sperm work. So, OPN could be used as a possible biomarker for Madura bull's fertility. Artificial insemination center in Indonesia is an institution for procuring frozen semen with SOP based on SNI 4869-1-2017 Frozen semen (Badan Standarisasi Nasional 2017). The bulls at the Lembang and Singosari AI centers have gone through standardization of the quality of the seeds contained in the Regulation of the Minister of Agriculture of the Republic of Indonesia Number 10/Permentan/PK.210/3/2016. The Indonesian National Standardization Agency in the document stated that the frozen semen in the Lembang and Singosari AI centers came from normal bulls who had been selected based on their lineage (pedigree/pedigree), production and reproductive abilities, healthy, and free of infectious

diseases according to the law. Therefore, in this study the possibility of sub-fertility and infertility due to inbreeding in the population of Madura cattle that we use is minimal.

OPN is derived from the accessory glands and is present on the entire surface of sperm membranes. Thus, sperm also carry OPN (Preedaa et al. 2020). OPN has been shown to have interactions with fibronectin, collagen, and numerous other types of integrins that may be found in cell membranes, Sertoli cells, tubular basement membranes, spermatid acrosomes, and spermatogonial stem cells. OPN which is formed in seminal plasma can connect to sperm and stay in the reproductive tract of the female until the fertilization process has taken place. In addition to this, OPN has the ability to atJejutach to oocyte integrins and plays a role in egg-sperm recognition (Rorie et al. 2016; Pilane et al. 2020). On the other hand, OPN mRNA has been found in bull seminiferous tubules; however, this OPN mRNA was only shown to be connected with particular stages of the seminiferous cycle (Chen et al. 2022). This suggests that OPN from the accessory gland and its synthesis in the seminiferous tubules may be controlled by events that regulate spermatogenesis and enhanced the functional features of the sperm, respectively. In this study, which demonstrated an excellent functional sperm characteristic and a strong association between OPN and sperm functional characteristics, fertility levels in highly fertile bulls were likewise high (Figure 1 and Table 2). The methods through which OPN improves sperm function are still largely unclear. However, its correlation with bovine ejaculates with increased freezing resistance (Jobim and Oberst 2004; Rego et al. 2016) supports the concept that OPN plays a role in sperm quality and fertility. The number of capacitated spermatozoa increased when bovine sperm was incubated with OPN (Monaco et al. 2009 and similar results were reported for buffalo sperm (Boccia et al. 2013). As capacitation occurs, sperm hyperactivate, which is an important phase in fertilization because it allows them to be released from the oviductal reservoirs and penetrate through the cumulus cells and the zona pellucida (Molina et al. 2018). As a result, larger amounts of OPN may cause hyperactivation, resulting in enhanced sperm total motility. According to Hernawati (2015), OPN is found on the surface of the plasma membrane of sperm and plays a function in the reduction of reactive oxygen species (ROS) generation. As a result, it was discovered in this study that the expression of the OPN gene showed a very significant association with motility. OPN produced by the accessory glands (Cancel et al. 1999) can permeate spermatozoa during ejaculation and persist until the spermatozoa reach the isthmus junction, which plays a role in sperm-egg recognition activities (Souza et al. 2009; Preedaa et al. 2020). In addition, OPN in buffalo semen improves sperm viability and acrosome integrity (Boccia et al. 2013). As a result, OPN has an indirect role in fertilization modulation.

In conclusion, we show that OPN is substantially expressed higher in sperm from Madura bulls categorized as highly fertile based on field conception rate, as indicated by its correlation with sperm quality and fertility levels. This study also revealed that OPN may be a useful marker for determining Madura bull fertility status. OPN mRNA expression studies in connection to field conception rates provide insight into the mechanisms behind the selective retention of particular transcripts during spermatogenesis and their importance in enhancing field fertility. Reproductive assessment and management tools are essential for identifying and selecting high-potential Madura bulls for AI programs, and their association with molecular markers will improve their utility.

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