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Differential Expressions of Protamine 1 (PRM1) and Protamine 2 (PRM2) Genes as Markers of Semen Quality in Pasundan Bulls

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ABSTRACT

Several gene expressions are related to the success of spermatogenesis. Protamine plays an important role in sperm DNA packaging, spermatogenesis, and sperm quality. This study aimed to isolate RNA from spermatozoa and determine the gene expression profiles of protamine 1 (PRM1) and protamine 2 (PRM2). Six Pasundan bulls aged 5-8 years with a body weight of 380 kg-430 kg were used for this study. The Pasundan bulls were classified into group A (70%-79%) and group B (80%-89%) based on their sperm motility in fresh semen. In this study, correlation analysis was performed between fresh semen characteristics (volume, motility, concentration, abnormality, viability, intact plasma membrane (IPM), DNA integrity) and frozen semen characteristics after thawing (motility, viability, IPM, and DNA integrity). Total RNA was isolated from the frozen sperm pellet, and cDNA was synthesized. Specific PCR primers were used for the transcription of PRM1 and PRM2 from sperm. PRM1 and PRM2 gene expressions were evaluated by qRT-PCR, and ACTB was used as a control. The results showed that progressive sperm motility in the fresh sperm positively correlated with sperm viability and IPM. PRM1 and PRM2 were higher ($p<0.05$) in group B than in group A. This condition indicates the existence and influence of protamine on the parameters of progressive sperm motility. The expression of PRM1 and PRM2 genes could use as markers of semen quality in Pasundan bulls.

Keywords: gene expressions; PRM1; PRM2; Pasundan bulls; progressive motility

INTRODUCTION

Pasundan cattle is one of the Indonesian genetic bull resources to be developed and conserved (Santoso *et al.*, 2021). Decree No. 1051/Kpts/SR.120/10/2014 of the Minister of Agriculture ordered the legalization of Pasundan cattle genetic resources in Indonesian livestock (Kementan, 2014). Pasundan cattle are more closely related to Ongole crosses (PO) in body size, while Madura cattle are more closely related in skull size (Sulasmi *et al.*, 2017). Pasundan cattle is an indigenous breed with advantages in maintenance efficiency, drought resistance, resistance to tropical diseases, and resistance to stress due to weather changes (Dwitresnadi *et al.*, 2015). Pasundan cattle can adapt to sheep as carriers of malignant catarrhal fever (MCF). Bulls with good semen quality play a role in female pregnancy but also accelerate the genetic improvement in artificial insemination (AI) (Diskin, 2018; Ramm, 2014). Indonesian National Standardization (SNI) number 4869.1: 2021 requires artificial insemination to have motility of at least 70% for fresh semen and at least 40% for frozen semen and a maximum of 20% abnormalities (BSN, 2021). Bull fertility is evaluated and selected by Breeding Soundness Examination (BSE). Barth (2018) explained that the capacity of sperm production could determine male fertility. Sperms are produced by epithelial cells in the tubular testes, called spermatogenesis (Senger, 2012). According to Ganguly *et al.* (2013), the expressions of various genes are related to the success of spermatogenesis. A previous study showed thousands of mRNA transcripts in the sperm (Ren *et al.*, 2017; Sun *et al.*, 2021).

Protamines (PRM) are nuclear proteins with low molecular weights in the sperm head. Protamine is the sperm replacing histone of somatic cells in many steps of spermiogenesis to promote structure formation and chromatin condensation of sperm to become more solid and compact (Dogan *et al.*, 2015). Protamine plays an important role in sperm DNA packaging (Dogan *et al.*, 2015), spermatogenesis, and sperm quality (Akmal *et*

al., 2016). The protamine 1 (PRM1) is a genetic marker that has been demonstrated by correlating fertility in crossbred Frieswal cattle (Ganguly *et al.,* 2013), *Bos taurus* (Fortes *et al.,* 2014; Dogan *et al.,* 2015; Parthipan *et al.,* 2017), and Aceh cattle (Helmi *et al.,* 2020). The protamine 2 (PRM2) study was reported in the crossbred Frieswal cattle (Ganguly *et al.,* 2013).

Routine semen evaluation at the Artificial Insemination Center is an inaccurate indicator for measuring the fertility level of the bull (Nasrin *et al.,* 2021). Protamine (PRM) is associated with male fertility as it is a nuclear protein found in the sperm head and plays an important role in sperm DNA packaging and spermatogenesis, and has been shown to correlate with bull fertility in *Bos taurus* (Parthipan *et al.,* 2017). Conventional quality assessment is considered inaccurate for predicting the fertility potential of the bull. Molecular evaluation becomes one attempt to identify the bull's fertility potential. The expressed genes related to sperm quality have not been previously reported and need confirmation, especially in Indonesian cattle such as Pasundan cattle. This study aimed to compare PRM1 and PRM2 gene expressions in different sperm motility stages of Pasundan bulls.

MATERIALS AND METHODS

Animals

The Animal Ethics Committee of the Faculty of Veterinary Medicine, IPB University (Ethics Approval No. 161/KEH/SKE/VII/2019) approved all procedures in this study. A total of six Pasundan bulls aged 5-8 years with body weight (BW) of 380 kg-430 kg were used in this study. All bulls were fed with a combination of fresh forage (10% BW) and concentrate (1% BW) twice daily (morning and evening times). Water was provided as *ad libitum*. Pasundan bulls with fresh semen motility of ≥ 70% (SNI number 4869-1: 2021) were divided into two groups, namely group A (A1-A3, sperm motility of 70%-79%) and group B (B1-B3, sperm motility of 80%-89%).

Evaluation of Fresh and Frozen Semen

Semen was collected each morning using an artificial vagina according to the Standard Operating Procedure (SOP) of the Ciamis-West Java Regional Artificial Insemination Center (RAIC). Immediately after collection, the semen was examined macroscopically and microscopically (Arifiantini, 2012). The characteristics of fresh semen, such as volume, sperm motility, sperm concentration, sperm abnormality, sperm viability, membrane integrity (IPM), and DNA integrity, confirmed the molecular studies. In this study, sperm motility, viability, membrane integrity, and DNA integrity were measured in frozen sperm after thawing. In addition, secondary data (macroscopically and microscopically) were obtained from the laboratory records, while primary data were collected during the experiment.

Semen was frozen using Tris-egg yolk (TEY), following the SOP of the insemination station for the preparation of frozen semen. Semen was equilibrated in a refrigerated chamber (5 °C) for four hours and then packed using the MPP Uno, automatic filling and sealing machine (Minitub, Tiefenbach, Germany). For ten minutes, freezing was performed in a liquid nitrogen vapor over a 60 x 40 x 30 cm^3 Styrofoam box. Frozen semen was then stored in a container with liquid nitrogen (-196 °C) for further studies (sperm motility, viability, membrane integrity, and DNA integrity). The parameters of fresh and frozen semen were then compared. The frozen semen used for sperm parameter analysis and RNA collection was from the same ejaculate as the fresh sperm used for the previous analysis.

Sperm motility was assessed using the Computer-Assisted Semen Analyzer at various stages, including fresh semen and semen after freezing and thawing (CASA; Andro Vision, Minitub Germany). Twenty-five microliters (25 μL) of semen were homogenized and diluted with 725 μL of 0.9% NaCl before being dropped on a glass slide and covered with a coverslip. Observations were performed using a 400x magnification microscope (Olympus, Tokyo, Japan). Sperm concentration (10^6/mL) was calculated using the Photometer SDM 6 (Minitub, Tiefenbach, Germany).

In this study, sperm viability was determined by mixing 20 μL of sperm with 80 μL of eosin-nigrosine dyes (1:4) on a sliding glass and viewing it under a microscope at 400x magnification. The intact plasma membrane (IPM) was examined by the hypoosmotic swelling (HOS) assay. Twenty microliters (20 μL) of thawed semen mixed in 1 mL of hypoosmotic medium (0.3 g fructose and 0.7 g sodium citrate in 100 mL distilled water) then incubated in a water bath (37 °C) for 30 mins (Arifiantini, 2012). The analysis was performed under a microscope with 400x magnification. DNA integrity was examined using a fluorescence microscope after staining with Acridine Orange (AO) (Yusrina *et al.,* 2018). Fresh semen preparations were conducted by fixation with a Carnoy solution of acetic acid and methanol (1:3). Observations were then performed under a fluorescence microscope (Olympus, Tokyo, Japan) with 400x magnification with 450-490 nm excitation light and a blocking filter of 530 nm under dark conditions.

Total RNA Isolation

The total RNA isolation was performed by thawing the frozen semen (1x10^8 sperm cells) at 37 °C for 30 seconds. Total RNA isolation was performed according to the modified RNeasy® Mini Kit Qiagen protocols. The process of wall and cell membrane lysis was assisted using a modified 20G-needle syringe. In this study, RNA elution was optimized by the repeated RNA elution step by re-applying the RNA elution product to the columnar spin membrane. Then, the RNA elution product was stored at -80 °C.

Table 1. Primers sequences to the presence of specific transcript in the RNA isolated from frozen sperm

Gene	Primer sequences	Product size (bp)	GenBank accession number
ACTB	F: 5′- CTTCCAGCAGATGTGGATCA -3′	136	NM_173979.3
	R: 5′- CGTTTTCTGCGCAAGTTAGG -3′		
PRM1	F: 5′- GACAGTAACCACACAGTAGC -3	136	NM_174156.2
	R: 5′- GTGGCATTGTTCGTTAGCAG -3′		
PRM2	F: 5′- GAGTCCAACTGAAAGTCCAC -3′	147	NM_174157.4
	R: 5′- CTCCTGTGTCTGTAGTGGTA -3′		

Note: F= Forward, R= Reverse.

Evaluation of the Quality of the Isolated Sperm RNA

The total RNA concentrations of the samples were determined using the Nano Drop spectrophotometer (Thermo Fisher Scientific, Inc., West Palm Beach, FL, USA). The absorbance value was used to measure the RNA concentration at 230, 260, and 280 nm wavelengths. The integrity of the isolated RNA was examined using the Agilent 2100 Bioanalyzer System (Agilent Technologies, USA) in Macrogen, South Korea.

Procedure of Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed by transcribing the RNA extract into complementary DNA (cDNA) using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania, EU) according to the manufacturer's protocols. Specific primers of the PRM1 gene, the PRM2 gene, and the β-Active housekeeping gene (ACTB) (Table 1) were designed using Primer3 software (Rozen & Skaletsky, 2000). The DNA amplification (peqSTAR 2X, Germany) was performed at the pre-denaturation temperature of 95 °C for 5 min. This step was followed by 35 cycles of the pre-denaturation phase at the same temperature for 10 seconds. Annealing at 60 °C for 20 seconds and 72 °C for 3 min. The PCR product was analyzed by 1.5% agarose gel electrophoresis.

Procedure of Quantitative Real-Time Transcriptase Polymerase Chain Reaction (qRT-PCR)

Quantification of cDNA by the qRT-PCR method was performed using the qTower3 G touch (Analytic Jena, Germany). The qRT-PCR process was performed with the following program: pre-denaturation at 95 °C for 60 seconds, 40 cycles for denaturation phase at 95 °C for 10 seconds, and extension at 60 °C for 30 seconds. Samples were analyzed in Duplo, where the delta Ct value (Δ_{Ct}) was calculated as the difference between the geometric mean of the target gene and the reference gene: $\Delta\Delta C_T = (C_{T,\ Target} - C_{T,\ house\text{-}keeping})_{Time\ x} - (C_{T,\ Target} - C_{T,\ house\text{-}keeping})_{Time\ 0}$, followed by calculation of $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001).

Statistical Analysis

The fresh and frozen semen characteristics and gene expression patterns of PRM1 and PRM2 between the two sperm motility groups were analyzed using the Student's t-test. Data were expressed as mean ±standard deviation (SD), as a significant difference was due to $p<0.05$. The correlation between parameters was analyzed with a linear regression using SPSS version 26 (IBM® SPSS® Statistics, United States).

RESULTS

The Pasundan bulls were grouped based on the progressive motility of fresh semen from 70%-79% (A) and 80-89% (B). The fresh and frozen semen parameters (Table 2) showed a significant positive correlation (Table 3) between fresh semen sperm motility and fresh semen viability (r= 0.860), frozen semen viability (r= 0.855), and frozen semen IPM (r= 0.841). There was also a positive correlation ($p<0.05$) between the viabilities of fresh semen and frozen semen (r= 0.917) and frozen semen IPM (r= 0.820).

The sperm RNA samples obtained from the Pasundan bulls in this study had an $OD_{260/280}$ value of 1.84-1.93. The concentration of RNA products isolated in this study ranged from 24.5 – 120.2 ng/µL (85.57±28.94 ng/µL). The average concentration of cDNA product was 2772.15±261.29 ng/µL with the spectrophotometry value of 1.71-1.73 for $OD_{260/280}$. The results of RT-PCR on

Table 2. Characteristics of fresh semen and frozen semen from Pasundan bulls in two different sperm motility groups (n= 6)

Variables	Groups	
	A	B
Fresh semen		
Volume of ejaculate (mL)	8.67±3.21	6.00±1.91
Sperm concentration (10^6/mL)	638.00±291.98	781.33±163.66
Progressive sperm motility (%)	75.30±3.61[a]	85.13±1.84[b]
Sperm abnormality (%)	11.12±1.91	10.24±2.08
Sperm viability (%)	84.82±2.62	88.83±3.52
Sperm membrane integrity/ IPM (%)	76.63±11.81	79.62±6.62
Sperm DNA integrity (%)	91.58±2.36	92.58±0.74
Frozen semen (post-thawed)		
Progressive sperm motility (%)	33.12±2.41	45.88±14.96
Sperm viability (%)	45.27±12.45	61.18±11.79
Sperm membrane integrity/ IPM (%)	51.51±2.35	58.69±4.60
Sperm DNA integrity (%)	87.04±1.86	89.53±2.10

Note: A= the sperm motility group of fresh semen 70%-79%; B= the sperm motility group of fresh semen 80%-89%. Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 3. Correlation between fresh and frozen semen of Pasundan bulls in two different sperm motility groups

	Mot.FS	Con.FS	Abn	Vib.FS	IPM.FS	Ig.DNA.FS	Mot.ZS	Vib.ZS	IPM.ZS
Con.FS	0.504	1							
Abn	-0.413	-0.818*	1						
Vib.FS	0.860*	0.594	-0.687	1					
IPM.FS	0.151	-0.710	0.746	-0.192	1				
Ig.DNA.FS	0.596	0.487	-0.064	0.492	-0.063	1			
Mot.ZS	0.614	0.611	-0.431	0.595	-0.076	0.394	1		
Vib.ZS	0.855*	0.660	-0.545	0.917*	-0.142	0.689	0.804	1	
IPM.SZ	0.841*	0.472	-0.640	0.820*	-0.062	0.222	0.299	0.609	1
Ig.DNA.ZS	0.369	-0.105	0.326	0.034	0.665	0.100	0.621	0.269	-0.007

Note: FS= Fresh semen, ZS= Frozen semen (post-thawed), Con= Concentration, Vib= viability, IPM=sperm membrane integrity, Ig.DNA= DNA integrity. Mark (*) shows a significant correlation (p<0.05).

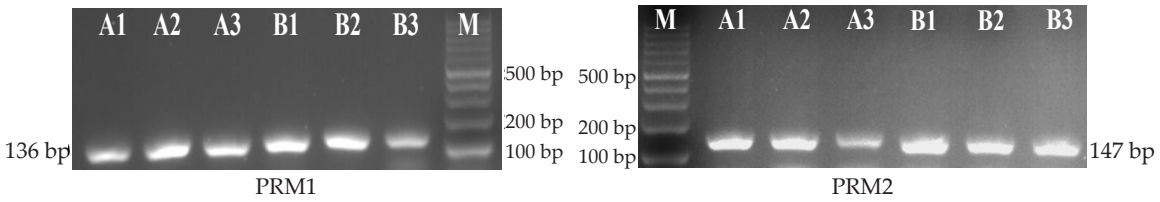

Figure 1. The amplification results of PRM1 and PRM2 genes in Pasundan bulls from two different sperm motility groups. M= 100 bp DNA marker, 1-6= amplified product sample, A1-A3= the sperm motility group of fresh semen 70%-79%, and B4-B6= the sperm motility group of fresh semen 80%-89%.

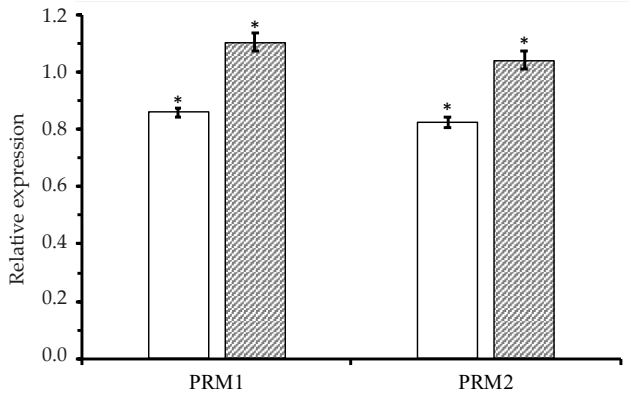

Figure 2. The expressions of PRM1 and PRM2 genes in Pasundan-bull sperms from groups A and B. Mark (*) shows a significant difference (p<0.05). A (□) = the sperm motility group of fresh semen 70%-79%; B (▒) = the sperm motility group of fresh semen 80%-89%.

mRNA abundance in ACTB gene (β-actin), PRM1, and PRM2 are shown in Figure 1. Confirmation of the specific RT-PCR product size of intended genes (PRM1 and PRM2) is presented in Figure 1. Measurement of gene expression (Figure 2) revealed a significantly different value (p<0.05) for the expression of PRM1 and PRM2 genes in groups A and B, respectively.

DISCUSSION

Progressive motility of fresh sperm is positively correlated with sperm viability and PMI in Pasundan bulls. A significant correlation between sperm motility and fertility was found by Januskauskas *et al*. (2003), with the correlation values ranging from 0.150–0.830. The highest correlation value (r= 0.830) was measured when sperm motility was combined with functional parameters, such as sperm movement pattern, DNA integrity, and viability (Januskauskas *et al*., 2001). Ganguly *et al.* (2013) reported a significant positive correlation between progressive sperm motility with sperm viability and IPM in fresh semen. Progressive sperm motility is widely considered the most reliable indicator of sperm motility for predicting the ability to fertilize and achieve pregnancy (Khalil *et al*., 2018).

Sperm motility occurs when sperm have a well-functioning membrane to generate kinetic energy (Sukmawati *et al*., 2014). A structural change in sperm membrane cells after thawing is associated with the ability to rotate the energy source through ATP and ADP conversions (Meyers *et al*., 2019). This change may affect mitochondrial function in cell metabolism and sperm function (Agarwal *et al*., 2014; Meyers *et al*., 2019; Indriastuti *et al*., 2020). The method used to optimize RNA isolation from frozen sperm increased RNA concentration. This study used a modified 20G needle to lyse the cell wall and membrane. The average RNA concentration obtained was higher than that was reported by Raval *et al*. (2019) in *Bos indicus* at 8.15 ng/μL.

In this study, the main difference in mRNA abundance of PRM1 and PRM2 genes in fresh sperm was found to be 70%-79% sperm motility in group A compared to 80%-89% sperm motility in group B. The averages transcript levels of PRM1 and PRM2 were higher in group B than in group A. A study on crossbred Frieswal cattle revealed a higher transcript level of PRM1 (p<0.05) in the group with high-quality sperm (progressive motility: 57.61±1.41%) than in the group with low-quality semen (progressive motility: 18.45±1.61%). The high mRNA expression of the PRM1

gene is due to its role in sperm DNA packaging and stabilization (Pardede *et al.,* 2022). The mRNA level of the PRM2 gene in crossbred Frieswal cattle showed no difference between high-quality and low-quality sperms. The abundance of PRM1 gene in motile sperm by sperm fraction separation was detected in *Bos indicus* (Raval *et al.,* 2019) and *Bos taurus* (Pardede *et al.,* 2022). Other results were reported by Parthipan *et al*. (2017) for *Bos taurus* that the PRM1 expression level showed no difference between high and low fertility groups.

PRM1 abundance may be influenced by fertility status (Pardede *et al.,* 2022). Different expression levels in PRM1 and PRM2 genes from the sperm mRNA occur in fertile and infertile males (Bansal *et al.,* 2015). The study results on infertile males suggest a link between abnormal histone and protamine ratio (Bao & Bedford, 2016; Wang *et al.,* 2019). Jodar *et al*. (2012) reported a significant decrease in mRNA in PRM1 and PRM2 genes in male sperm with sperm motility disorder (as the no zoospermia). Moghbelinejad *et al*. (2015) also found lower PRM1 and protein transcriptions from the testis biopsy results of infertile males.

Abnormal protamine expression may be caused by abnormal transcription and translation that modulate regulatory functions affecting protamine and other genes involved in spermatogenesis (Ganguly *et al.,* 2013). Another study reported the decreased total amount and localization of PRM1, resulting in structural deformation of spermatids and impaired fertility in bulls (Dogan *et al.,* 2015). In addition, protamine expression could be an indicator of spermiogenesis, as abnormal protamine expression could increase the number of dead cells (Dehghanpour *et al.,* 2017). Jodar *et al.* (2015) and Selvaraju *et al.* (2017) state that the spermatogenesis process, sperm function, and fertility success can be determined by the level of sperm RNA expression.

Research on genomic analysis related to PRM1 and PRM2 genes in Pasundan bulls needs to be continued either partially or completely with larger samples. Further studies are needed to determine the possible molecular mechanism underlying the reduction of PRM1 and PRM2 transcripts in the sperm from movement disordered bulls.

CONCLUSION

Measurement of gene expression revealed a significantly different value for the expression of PRM1 and PRM2 genes in groups A and B, respectively. The PRM1 and PRM2 gene expressions can be used to predict the quality of fresh and thawed semen from Pasundan bulls. Progressive sperm motility of fresh sperm is positively correlated with sperm viability and PMI.

CONFLICT OF INTEREST

Asep Gunawan and Cece Sumantri serve as editors of the Tropical Animal Science Journal, but have no role in the decision to publish this article. The authors also declare that there are no financial, personal, or other relationships with any other person or organizations related to the material covered in the manuscript.

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