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Research Article

The Sperms Post-Thawing Quality and Proteomic Seminal Plasma on Fertility Performance of Bali-Polled Bull

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Abstract | The development of local bull in many regions is currently leading to the development of local bulls and Indonesia is no exception, especially in South Sulawesi Province. One of local bulls in which developed for approximately last ten years is Bali-polled bull. Thus, the selection of polled bulls is crucial, mainly in modern livestock management. The aims of this study to identify sperms post-thawing quality and proteomic plasma semen as fertility performance in Bali-polled bull. The utilization of Bali-horned bulls as a point of reference was necessary to achieve this aim. The semen samples from Bali-horned and Bali-polled bulls were obtained twice weekly using an artificial vagina. The semen samples were immediately sent to the laboratory for processing. Parameters measured were sperm motility, abnormality, viability, acrosome integrity, and membrane integrity. Liquid chromatography-mass spectrometry (LCMS/MS) analysis was used to assess the confirmation profile protein of seminal plasma. The conception rate from artificial insemination was used to determine the fertility rate (AI). This investigation revealed that the quality of the sperms from Bali-polled bulls and Bali-horned bulls did not substantially change after thawing ($p > 0.05$). However, sperm acrosome integrity was considerably ($p < 0.05$) higher in Bali-polled bulls than in Bali-horned bulls. In the profiling protein seminal plasma, ZPBP protein expression was found in the Bali-polled bull which was not found in the Bali-horned bull. Bali-polled bulls and Bali-horned bulls did not differ significantly ($p > 0.05$) in terms of fertility rate on conception rate. In conclusion, sperms post-thawing quality of Bali-polled bull have a good category according SNI 4869-1:2017 and proteomic seminal plasma of Bali-polled bull has a protein profile linked to reproductive function.

Keywords | Bali-polled bull, Sperm, Thawing, Proteomic, Fertility

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INTRODUCTION

The development of local bull in many regions is currently leading to the development of local bulls and Indonesia is no exception, especially in South Sulawesi Province. One of local bulls in which developed for approximately last ten years is Bali-polled bull. Generally, Bali cattle as local cattle in this region has a pair of horns, however it is become unique because several years ago, it was found Bali cattle without any horn. By definition,

polled bull are bull whose horns do not grow naturally (Baco et al., 2020).

Polled cattle have some benefits, such as lowering the danger of injuries that often occur in breeders due to horns, preventing bruising on the carcass and deterioration to the skin, and preventing the carcass from bruising (Mueller et al., 2021). Utilizing genetic selection to increase the number of polled (hornless) cattle is an alternative to dehorning. Horns are inherited as an autosomal recessive characteristic (Long and Gregory, 1978).

There is a chance that Indonesia will utilize artificial insemination to produce Bali-polled cattle. Thus, the selection of polled bulls is crucial, particularly in contemporary livestock management (Brockmann, 2000). The development of reproductive biotechnology for livestock has facilitated the exploration of cattle's reproductive performance, population growth, and genetic quality. Artificial insemination, the first generation of reproductive technology, aims to successfully utilize large bulls, reduce the spread of reproductive diseases, and improve the genetic quality of animals (Said, 2020).

The success of artificial insemination (AI) is largely determined by the bull factor used in the artificial insemination station. Even while all cows equally contribute to conception success or failure during AI, the bull plays a crucial role because not all bulls have the same fertility rates. The ability of bulls to resisted the cryopreservation of their sperm varies. Recent research has indicated that this may impact fertility (Harayama et al., 2010; Kumaresan et al., 2012). Therefore, it's crucial to understand sperm quality when trying to conceive.

Fertility assessment methods that have been developed for a long time are using the BSE method (Thundathil et al., 2016) which still need to be combined with several other parameters such as semen quality analysis (Boe-Hansen et al., 2015). The difference in spermatozoa motility is related to the plasma semen proteins that can be found together in the ejaculate (Netherton et al., 2018). Currently, fertility assessment has focused on identifying fertility marker proteins such as proteomic analysis of goods derived from semen plasma (Gomes et al., 2020). Numerous proteins have been involved in regulating spermatozoa quality via their participation and expression (Wang et al., 2019). However, there is currently limited knowledge about the post-thawing quality of sperms and proteome plasma seminal of Bali-bull polled. Therefore, more investigation is required to establish the post-thawing sperm quality and proteomic plasma seminal linked with bull fertility in Bali-polled bulls. This study aimed to identify sperms post-thawing quality and proteomic plasma semen as fertility performance in Bali-polled bull. Thus, finding new potential Bali-polled bull as a source of quality beef with higher value and which will economically impact Indonesian livestock.

MATERIALS AND METHODS

The study was conducted at Samata integrated Farming System, Gowa, Indonesia and the Laboratory of Animal Reproduction, Semen Processing Unit, Faculty of Animal Science, Hasanuddin University, Makassar, Indonesia. Profiling proteins of plasma seminal was analyzed at the Research Center for Applied Zoology, National Research and Innovation Agency, Cibinong, Indonesia. Fertility test was performed at Lappariaja, Bone, Indonesia from February to October 2022. The bulls used in this study from Faculty of Animal Science Hasanuddin University, Makassar and Samata integrated Farming System, Gowa, Indonesia. The semen samples were obtained from a Bali-polled bull aged 8 years with body weight of 320kg and a Bali-horned bull aged 5 years with body weight of 300kg. The bulls were kept in a barn with individual stalls and were given concentrate (20%) and elephant grass (80%) in the morning and evening. The Animal Ethics Committee of Hasanuddin University, Makassar, Indonesia, approved all procedures in the present study. The utilization of Bali-horned bulls as a point of reference was necessary to achieve this aim.

Semen collection and Processing Semen

The Semen samples from Bali-polled and Bali-horned bulls were taken twice weekly throughout 5 successively utilizing an artificial vagina. The semen samples were processed in the lab as soon as they were obtained. The sperm was processed as described by Diansyah et al. (2022[a]), with minor adjustments. Following the AI Center's Standard Operating Procedure for manufacturing frozen sperm using a commercial extender, the semen of Bali-horned and Bali-polled bulls was frozen (Andromed, Germany). The semen was adjusted for 4 hours at 5 °C. Using a Styrofoam box and liquid nitrogen vapour above, The frozen semen completed the cryopreservation procedure for 10 minutes. The frozen sperm was placed in a -196°C liquid nitrogen container.

Thawing and Evaluation

The frozen semen was thawed by inserting the straw into a thawing device (Minitube, Germany) for 30 seconds. Tissue paper was used to dry the straw before being chopped off at the ends and placed into a microtube (Diansyah et al., 2020). The samples assessed were the motility, abnormality, viability, acrosome integrity, and membrane integrity of sperms.

Motility, Abnormality, and Viability of The Sperms

The motility of sperms was determined by placing 10 µl of semen on the object glass. CASA (Program Vision VersionTM 3.7.5 Minitube, Germany) was then used to analyse the semen (Diansyah et al., 2022a). The abnormality and viability of the sperms were evaluated by combining 10 µl of semen and 10 µl of Eosin 2% in the object glass. After drying, the object glass was examined using Indomicro View 3.7 software and a trinocular microscope (Primo Star, Zeiss, Germany). Red spermatozoa were categorized as dead, while colourless spermatozoa were believed to be alive. At least 200 sperm cells per observation were counted to provide an accurate calculation. Spermatozoa with cut tails, shattered tails, and abnormal head shapes were judged abnormal.

Acrosome Integrity

Acrosome integrity was determined by combining the test semen with a formol-saline solution (physiological NaCl solution with 1% formalin) in a ratio of 1:4. The inspection was conducted using microscope (Zeiss, Germany). A black head tip shows the proportion of acrosomes with intact acrosomal hoods.

Membrane Integrity

The membrane integrity was assessed microscopically by placing 10 µl of semen in HOST solution (0.179g NaCl in 100 ml of aquabides). The solution was then incubated for 30 minutes at 37°C. The investigation was carried out with a trinocular microscope (Primo Star, Zeiss, Germany). Sperms with intact membranes had circular tails, whereas sperms with straight tails were considered damaged.

Confirmation profiling protein of seminal plasma

Confirmation profiling protein of seminal plasma was determined by LCMS/MS (Liquid Chromatography Mass Spectrometer) analysis following Baharun (2021) and Diansyah et al. (2022b) methods with the minor modifications. Approximately 3–4 ml of the semen were centrifuged for 30 minutes at 6500 rpm. In a cryo box, a microtube containing the centrifuged supernatant was preserved at -20°C. 1D-SDS-PAGE was used to characterize the seminal plasma protein (SMOBIO TM® Technology, Inc., Taiwan). Coomassie Brilliant Blue stain (Sigma-Aldrich®, United States) were used for stanning the gels. Peptide digestion was used to carry out the protein bands that were carried out on the gel. Then, using a solution consisting of 2% ACN, 98% ultrapure water, and 0.1% formic acid, peptide fractionation was performed using a Nano LC Ultimate 3000 Series System Tandem Q Exactive Plus Orbitrap HRMS (Thermo Scientific, Bremen, Germany). Signal peptides were identified using a Thermo Scientific LTQ-Orbitrap mass spectrometer, 2002000 m/z.

Fertility Rate

The artificial insemination (AI) conception rate was used to calculate the fertility rate, as described by Pardede et al. (2020). The conception rate (CR) is the ratio of the number of inseminated cows to the number of pregnant cows after one insemination treatment to determine the reproductive rates of Bali-polled and Bali-horned bulls, 50 receptor cows were used. A transrectal pregnancy diagnosis was made 45 to 60 days after AI.

Statistical analysis

ANOVA was used to compare each parameter of sperms post-thawing quality. Fertility rate was analyzed using Chi-square. The p-value had to be less than 0.05 for the parameter to be considered significant.All analysis was performed using SPSS Version 25. Determining profiling protein composition using dataset of BioinfoGP (https://bioinfogp.cnb.csic.es/). Determining profiling protein pathway using dataset of Panther (https://pantherdb.org/) and Uniprot (https://uniprotorg/). Determining interaction between proteins using dataset of STRING (https://string-db.org/) (Szklarczyk et al., 2015).

RESULTS AND DISCUSSION

The Sperms Post-Thawing Quality of Bali-Polled Bull

The fertility rate can be predicted by microscopical analysis of the sperm post-thawing quality of Bali-polled bulls. The sperms post-thawing quality of Bali-polled bull post-thawing regarding motility, abnormality, viability, acrosome integrity, and membrane integrity are summarized in Table 1.

The post-thawing sperm quality of sperm from Bali-polled and Bali-horned bulls is displayed in Table 1. According to statistical analysis, the sperm motility of the Bali-polled bull and Bali-horned bull (49.5% vs. 49.0%, respectively) did not differ substantially ($p > 0.05$). Furthermore, there were no significant difference in sperm abnormality (13.9% vs. 14.1%), sperm viability (51.3% vs. 50.1%), or sperm membrane integrity (50.7% vs. 49.5%) ($p > 0.05$). However, sperm acrosome integrity was substantially ($p < 0.05$) higher in the Bali-polled bull (50.7% vs. 48.7%) than in the Bali-horned bull. This difference seems to not biologically affect the fertility level in the two bulls. The causes of this difference in the present study were not fully understood.

The Bali-polled bull in this study has a standard category of sperm post-thawing quality (Table 1). About the Indonesian National Standardization 4869-1:2017 for frozen

Table 1: The sperms quality post-thawing of Bali-polled and Bali-horned bulls

Bali Bull	Parameter				
	Motility (%)	Abnormality	Viability	Acrosome Integrity	Membrane Integrity
Polled	49.51.2	13.91.3	51.31.39	50.7[a]1.1	50.71.4
Horned	49.00.8	14.11.4	50.11.37	48.7[b]1.4	49.50.9

Means in a column with different superscripts differ significantly ($p < 0.05$).

bull semen (Rosyada et al., 2021), the sperm quality of the Bali-polled bull in this study was designated a normal category. It can be utilized for artificial insemination (AI) with at least 40% motility sperm.The statistical difference in acrosome integrity can be caused by the freezing and thawing processes during this procedure, sperms were exposed to several potential risks, including acrosome loss (Khalil et al., 2018) and a change in the integrity of its chromatin (Mukhopadhyay et al., 2011).

Kutchy et al. (2019) found that characteristics, including sperm motility and acrosome integrity, are related to bull fertility (Kumaresan et al., 2017). Viability and acrosome integrity are essential indicators in identifying between bulls with varied fertility (Bernecic et al., 2021). Acrosome integrity is vital for successful fertilization, whereby the overlying plasma membrane fuse and the outer acrosomal to cause a release of lytic enzymes either just before or upon contact with the zona pellucida surrounding the oocyte (Ickowicz et al., 2012). Therefore, if the acrosome prematurely reacts or is damaged during cryopreservation or soon after insemination, the potential for spermatozoa to successfully fertilize will be reduced (Thundathil et al., 1999). However, some additional analysis is needed to predict the fertility rate of a bull.

Profiling protein seminal plasma of Bali-polled bull

The proteomic seminal plasma of Bali-polled bull was analyzed using LCMS/MS to predict the fertility rate. The profiling protein seminal plasma of Bali-polled bull regarding protein seminal plasma compositions and protein-specific seminal plasma linked to reproductive function of Bali-polled bull are summarized in Figure 1 and Table 2.

Figure 1 shows the results of the LCMS/MS analysis of the protein seminal plasma compositions of the Bali-polled and Bali-horned bull with a MW between 12 and 65 kDa. A total of 196 proteins were identified in the Bali-horned and Bali-polled bull. A total 145 protein profiles are the same in plasma seminal from Bali-polled and Bali-horned bulls, which is the same proportion of the total protein composition (75.1%). In the Bali-polled bull was identified 27 protein profiles (14%) which were not identified in Bali-horned bull. While, in the Bali-horned bull, 21 protein profiles (21%) were identified but not identified in Bali-polled bull.

The proteomic seminal plasma of the Bali-polled bull was identified 172 protein profiles (Figure 1). Some of these proteins have reproductive functions such as sperm motility, signaling receptor activity, sperm-egg identification, response to oxidative stress, sperm protection, capacitation of sperm, freezing capability, signaling caspase activity and growth factor activity. In other breeds, 87 protein profiles were identified in the Simmental bull (Baharun, 2021) and 241 protein profiles were identified in the Sahiwal bull (Sharma et al., 2022).

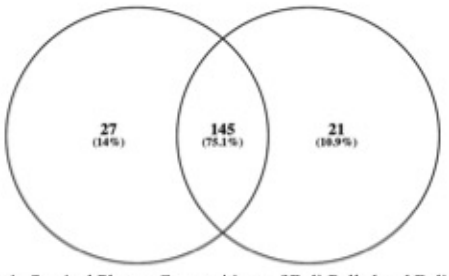

Figure 1: Protein seminal plasma compositions of Bali-polled and Bali-horned bulls

The results of Panther and Uniprot analysis (Table 2) show that the following proteins are involved in reproductive function in Bali-polled and Bali-horned bulls are TEXT101, FLOR3 (Folate Receptor Alpha 3), FLOR1 (Folate Receptor Alpha 1), BSP1, BSP3, BSP5 (Binder Sperm Protein), SPADH2 (Spermadhesin 2), PRSS55 (Serine Protease 55), ELSBPB1 (Epididymal Sperm Binding Protein 1), CRISP1 (Cysteine-Rich Secretory Protein 1), GPX3 (Glutathione Peroxidase 3), RNASE4 (Ribonuclease 4) and NGF (Beta-Nerve Growth Factor). Meanwhile, ZPBP protein expression was found in the Bali-polled bull which was not found in the Bali-horned bull. The Bali-polled bull discovered a protein profile that the Bali-horned bull did not (Table 2). The protein has the protein id ZPBP and accession number F1N369 (Zona Pellucida Binding Protein). The ZBPB protein (F1N369) contains 325 amino acids. Figure 2 shows the structure of the ZPBP protein interaction.

The results of STRING analysis (Figure 2), the ZPBP protein has interactions with CFAP61, SPATA16, ZAN, VWC2, and TSACC. The ZPBP protein has co-expres

Table 2: The protein specific seminal plasma of Bali-polled and Bali-horned bulls related to reproductive function

Protein	Accession Number	Function	Bali Bull	
			Polled	Horned
TEX101	A6QPE3	Motility sperm	+	+
FOLR3	P02702	Signaling receptor activity	+	+
FOLR1	E1BJL8	Sperm-egg recognition	+	+
ZPBP	F1N369	Binding sperm to zona pellucida	+	-
BSP 1	P04557	Motility sperm	+	+
BSP 3	P81019	Capacitation sperm	+	+
BSP 5	P02784	Freezing capability	+	+
SPADH2	P82292	Motility sperm	+	+
PRSS55	E1BLW6	Motility sperm	+	+
ELSPBP1	E1B9P4	Capacitation sperm	+	+
CRISP1	E1BC47	Sperm protection	+	+
GPX3	P37141	Response to oxidative stress	+	+
RNASE4	Q58DP6	Signaling caspase activity	+	+
NGF	P13600	Growth factor activity	+	+

+: Protein expressed, -: Protein non-expressed

Figure 2: The structure of ZPBP protein interaction

sion with CFAP61 and SPATA16. The ZBPB protein (F1N369) has interactions with several proteins (Figure 2) including *Monodelphis Domestica* (Nolte et al., 2019) having protein interactions for co-expression with the CFAP61 protein (Cilia And Flagella Associated Protein 61), in *Rattus Norvegicus* and *Mus Musculus* (Crapster et al., 2020) have interactions on the SPATA16 protein (Spermatogenesis Associated 16), in humans (Zhang et al., 2018) have interactions on TSACC (TSSK6-activating co-chaperone protein), on the boar (Feugang et al., 2018) have interactions on the ZAN protein (Zonadhesin), and on the alpaca (Richardson et al., 2019) have interactions on the VWC2 protein (Von Willebrand Factor C Domain Containing 2). However, no information or reports have been found regarding the ZPBP (F1N369) in Bali-polled bulls.

Based on Uniprot analysis, gene ontology of the ZPBP protein (F1N369) was found to be classified according to the function of cellular components and biological processes. The ZPBP protein (F1N369) is expressed in cellular components with a function in sperm mediation with the zona pellucida while in biological processes it is expressed in the function of acrosome formation and the zona pellucida, where sperm attach (Gaudet et al., 2011).

Proteins have been identified in sperm motility and metabolism, membrane remodeling and function, resistance and preservation against ROS, capacitation, and acrosome responses expressed in the fluid surrounding sperm cells in semen (Moura et al., 2018). Semen plasma contains many sperm-binding proteins that modify the sperm membrane's structure and function. Studies show that the complex role played by semen plasma proteins in controlling sperm activity is expressed during several different cellular events. There is substantial evidence that sperm surface-bound seminal plasma proteins influence sperm behavior and function (Purdy, 2006).

The Fertility Rate of Bali-Polled Bull

The fertility rate of Bali-polled bull in the present study was used conception rate after artificial insemination (AI). The conception rate of Bali-polled bull is shown in Figure 3.

Figure 3 shows the Bali-polled and Bali-horned bull conception rates. According to statistical analysis, the conception rate of Bali-polled bulls and Bali-horned bulls (56% vs. 56%) did not differ substantially ($p > 0.05$). Bulls have greater fertility rates than cows since a bull could generate up to forty cows through natural reproduction or a large number with artificial intelligence (AI) algorithms (Kastelic, 2013). Conception rates become a suitable metric to

assess the efficacy of AI mating (Anggraeni et al., 2016). The Bali-polled bull in this study falls into the excellent fertility category (Figure 3). In Bali-polled bull, the CR-based fertility rate was 56%. In other breeds CR, Brahman bull was 55.3% (Islam et al., 2019) and Friesian Holstein bull was 45% (Anggraeni et al., 2016).

Fertility rate can be associated with sperms post-thawing quality. The sperms post-thawing quality of the Bali-polled bull in this study with motility by 49.46%, viability by 51.3%, and acrosome integrity by 50.68% (Table 1) had a fertility rate by 56% (Figure 3). López-Gatius (2012) revealed post-thawing of exotic beef bull frozen semen gave good the achievement for AI mating of motility by 38.0%, viability by 45.2%. Yániz et al. (2021) reported high fertility bulls have 46% of acrosome integrity. The acrosome is crucial in the fertilization process.

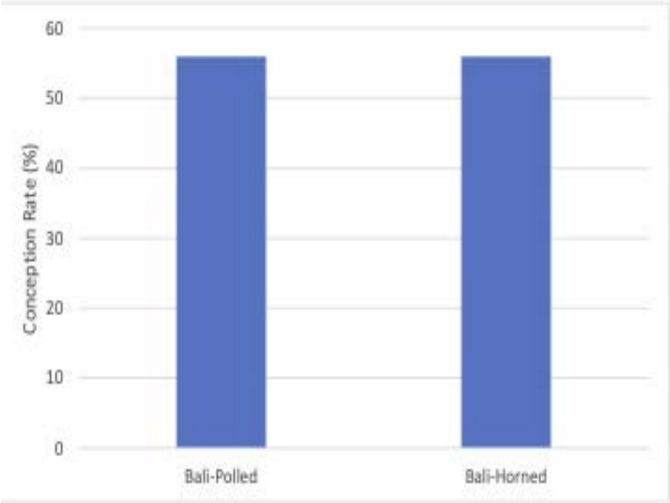

Figure 3: The conception rate of Bali-Polled and Bali-horned Bulls

The acrosome response is activated when spermatozoa come into contact with the zona pellucida, which releases and activates the enzyme of acrosome and allows the sperm to enter the zona pellucida (Miranda et al., 2009). In line with that, in Bali-polled bull was identified ZPBP protein whose role in binding sperm to the zona pellucida. This suggests that the protein discovered in Bali-polled bull relates to reproductive rate. ZPBP is one of the numerous proteins that assist in supplementary binding among acrosome-reacted sperm and the zona pellucida, an extracellular matrix unique to eggs (McLeskey et al., 1998). Furthermore, ZPBP is detected in the acrosomal matrix, which was implicated in the initial binding of the sperm acrosome to the zona pellucida and enhanced substantially during sexual maturity (Song et al., 2010).

ZPBP in mice was described by Lin et al. in 2007. Loss of ZPBP resulted in male infertility with abnormally round-headed sperm morphology and no forward sperm motility. As a result, the connections between the Sertoli spermatids and acrosomes were broken. Acrosomal membrane invaginations in males lacking ZPBP were intermittent, sub-fertile, and generated dysmorphic sperm that had a limited ability to cross the zona pellucida. ZPBP coevolved to perform joint roles in spermiogenesis.

CONCLUSIONS

The quality of sperms and proteomic seminal plasma can be used as a prediction for performance fertility in Bali-polled bulls. Sperms post-thawing quality of Bali-polled bull have a good category according to SNI 4869-1:2017 and proteomic seminal plasma of Bali-polled bull has a protein profile linked to reproductive function.

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CONFLICT OF INTERESTS

The authors stated that there were no competing interests.

ETHICAL CONSIDERATION

The authors have proven ethical problems such as plagiarism, information fabrication, misconduct and/or falsification, permission to publish, duplicate publication and/or submission, and redundancy.

NOVELTY STATEMENT

The present study highlights the possibility of predicting fertility performance of Bali-polled bulls using the quality of sperms and proteomic of seminal plasma.

AUTHORS' CONTRIBUTION

The study was carried out by AMD, MY, ALT, and MIAD, and all authors contributed equally. In addition, H and AB made essential contributions to the organization and analysis of the data for this work. All authors above consented to be held responsible for all parts of the work and participated in its preparation, drafting, and revision. They also

provided final permission for the version that was published.

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