Morphology and Morphometric of Frozen-thawed Pasundan Cattle Sperm Incubated in Extreme pH Media

Rini Widyastuti[1,2], Diah Nugrahani Pristihadi[1], Noer Muhammad Dliyaul Haq[1], Sigit Prastowo[3, 4], Hera Maheshwari[1], Cece Sumantri[5], and Arief Boediono[1*]

[1]*Department of Anatomy, Physiology, and Pharmacology, Faculty of Veterinary Medicine, IPB University, Bogor, Indonesia*
[2]*Laboratory of Animal Reproduction and Artificial Insemination, Department of Animal Production, Animal Husbandry Faculty, Universitas Padjadjaran. Jl. Raya Bandung- Sumedang Km. 21, West Java, Indonesia*
[3]*Department of Animal Science, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Indonesia*
[4]*Animal Breeding, Reproduction, and Biostatistics Research Group (ABRE-RG), Universitas Sebelas Maret*
[5]*Departmentof Animal Production, Faculty of Animals Science, IPB University, Bogor, Indonesia*
[*]*Corresponding author. Email: ab@apps.ipb.ac.id*

ABSTRACT
An evaluation of sperm morphology and morphometry is useful to predict sperm quality and in the diagnosis of fertile animals. The purpose of this study is to assess the morphology and morphometry of frozen-thawed Pasundan Cattle sperm, as well as the correlation between the two parameters. The sample was obtained from frozen-thawed Pasundan Cattle sperm incubated in pH 4 as acidic media, pH 7.2-7.4 as a control, and pH 11 as alkali media. After 5 minutes of incubation in media, all samples were returned to pH 7.2-7.4 by adding buffer media and immediately evaluated. The results revealed that the percentage of sperm with normal morphology was significantly lower ($p<0.05$) in the alkali or acidic groups compared to the control. Furthermore, the width of all sperm areas was significantly different in acidic compared to the control and alkali groups ($p<0.05$). There was no significant difference in treatments or control groups for the entire head and neck area morphometric ($p<0.05$). Moreover, the width of the sperm tail area displayed the same pattern as all widths of the sperm area. Furthermore, it can be concluded that extreme acidic and alkali media significantly impact on morphometry and morphology of frozen-thawed Pasundan Cattle sperm.

Keywords: *sperm, acid, alkaline, morphology, morphometry, Pasundan Cattle.*

1. INTRODUCTION

Pasundan Cattle is a Sundanese icon in West Java, Indonesia, which has superior characteristics. It has a small body size, good reproductive performance, and carcass proportion in rounds 52-55%. It has a distinct reddish coat color, and for bulls, it changes to black during sexual maturity due to the androgen hormone [1]. Pasundan cattle, like other local cattle in Indonesia, is relatively resistant to parasitic disease and adaptable to extreme environments, including poor feed conditions. Despite becoming a Sundanese icon, the population of Pasundan Cattle has been declining year by year, with only around 28,000 remaining in 2015 which spreads along the southern coast of West Java and the buffer zone area along the northern Priangan [2]. Based on these circumstances, it is critical to converse the Pasundan Cattle from extinction [3]. One method of conserving Pasundan Cattle is in-situ conservation by preserving their natural habitat, or ex-situ conservation by preserving the diversity of their habitats.

For *ex-situ* conservation, using frozen semen for artificial insemination or *in vitro* embryo production

may be an important method for increasing the population of the Cattle. Frozen sperm is commercially used all over the world for artificial insemination (AI) or *in vitro* embryo production. Moreover, Pasundan Cattle frozen sperm has been established in Indonesia and is widely used for AI by Indonesian local farmers. Frozen sperm will be exposed to various environmental conditions during the manufacturing process, distribution, and finally thawing for direct use in AI or *in vitro* embryo production. The changes in environmental conditions such as temperature and pH would lead to changes in reactive oxygen species (ROS) levels due to alteration in metabolic activities [4]. Previous research found that external pH has a significant impact on the motility, viability, and capacity of sperm [5] and it is critical for sperm motility [6]. When the sperm is exposed to an environment (media) with low pH or high pH, it may experience a detrimental effect on its cellular and molecular level [7].

There is a lack of information on the effect of extreme pH media on morphology and morphometry of freeze-thaw Pasundan sperm studies during *in vitro* conditions. A more investigation is necessary. Sperm morphology and morphometry are two of the quality parameters used to determine fertility. The purpose of this study is to assess the morphology and morphometry of frozen-thawed Pasundan cattle sperm, as well as the correlation between the two parameters after short time incubation in extreme pH media.

2. MATERIALS AND METHODS

2.1. Semen Sample and Sperm Preparation

The study was conducted with commercial frozen semen samples from 2 Pasundan bulls with the same batch number obtained from Lembang Artificial Insemination Center (Lembang, Indonesia). All the procedures related to this study were approved by the ethics committee of the Faculty of Veterinary Medicine, IPB, Indonesia (No:033/KEH/SKE/VI/2021). Three different solutions with different pH values were prepared by adding 5 M HCL or 5 M NaOH to buffer media (G-MOPS) just before the experiment. pH 4 represented an extreme acidic condition, while pH 11 represented an alkali condition while for the control group G-MOPS with pH 7.2-7.4 was prepared in this study.

In each experiment, frozen sperm were thawed in the water bath at 37°C for 10 seconds, pooled, and then diluted (1:1) with G-MOPS (Vitrolife, Swedeen) following centrifugation at 500 g for 5 minutes to remove the cryoprotectant. The supernatant was then discarded, and the pellet was re-suspended with 0.5 mL G-MOPS and divided into three equal aliquots. Each aliquot was then diluted in the specific pH media (treatment). The addition of sperm samples slightly altered the original buffer solution's pH, therefore we prepared buffer such that it always indicates the pH of the final sperm sample buffer mixture. Each sample was incubated for 5 minutes with a specific pH media at room temperature. Then, it was immediately returned to pH 7.2-7.4 by buffer solution addition. The assessment of sperm morphology was performed after 10 minutes of incubation at room temperature.

2.2. Evaluation of Sperm Morphology

Sperm morphology was determined by evaluating the number of normal versus abnormal sperm per 200 sperm in the eosin-nigrosine-stained smear with microscopy at 1000x magnification. A total of 200 sperms were examined for morphological abnormalities based on the previous method [8].

2.3. Morphometric Measurements of Sperm

Morphometric measurements of sperm were performed using the Scanning image analysis system (Computer Scanning Systems) connected with an Olympus IX70 microscope at 6.000 x magnification (100 x oil immersion objective). In this study, the sperm measurements were evaluated using software namely OLYMPUS FLUOVIEW ver. 4.2a. The width area of the sperm head, neck tail, and all of the sperm surface was measured.

2.4. Statistical analysis

All the treatments in this study were performed six times. Data were then analyzed using GraphPad Prism (version 8; GraphPad Software Inc., La Jolla, CA). The Shapiro-Wilk test was used to determine the data normality, and comparison between the group was compared using analysis of variance (ANOVA) followed by post-hoc test (Tukey test). The significance level in this study was set at $\alpha < 0.05$.

3. RESULTS

The sperm morphology measurements from respective treatments are shown in Table 1. The number of sperm with abnormal morphology increased threefold in the treatment group versus the control group ($p<0.05$). In both media at pH 4 and pH 11, the most common abnormality found is abnormal head morphology. Furthermore, when compared to controls, sperm with abnormal head morphology increased threefold in pH 4 and fourfold in pH 11 ($p<0.05$). When compared to the control, sperm with abnormal mid-piece and tail were not significantly different in any of the group treatments ($p>0.05$).

Table 1. The percentage of sperm with abnormal morphology after a brief incubation in different pH of media

pH media	Sperm with abnormal morphology (%)	Part of sperm with abnormal morphology (%)		
		Head	Neck	Tail
4	45.33±2.78[a]	39.43±1.71[a]	2.94±1.39	2.95±1.02
7.2-7.4	14.71±0.70[b]	10.58±1.03[b]	2.55±1.51	1.57±0.53
11	47.35±4.48[a]	40.72±3.98[a]	4.61±1.45	2.03±0.78

Data presented as average±SD. The different superscripts in the same column showed a significant difference

Table 2. Wide surface area of frozen-thawed Pasundan Cattle sperm after a brief incubation in different pH of media.

pH media	Sperm wide-area (μm^2)			
	All	Head	Neck	Tail
4	112.30±19.18[a]	42.57±5.35	17.61±3.37	45.62±11.39[a]
7.2-7.4	102.79±16.81[b]	42,28±4.04	17.67±3.79	54.51±12.43[b]
11	116.71±15.33[a]	42.07±3.66	17.08±3.13	50.34±10.55[c]

Data presented as average±SD The different superscripts in the same column showed a significant difference.

The parameters for the morphometric characteristics of sperm incubated in extreme acidic, extreme alkali, and control groups are summarized in Table 2. There is none group of treatment which have significantly different for the wide area of the head and the mid-piece compared to control ($P>0.05$). Interestingly, both groups of treatment have a smaller all surface and tail area compared to control ($p<0.05$).

4. DISCUSSION

Sperm is considered normal if the shape, size, and proportions of the head, mid-piece, and tail fall within the classification for the given species. Sperm morphology research is regarded as an important component of sperm analysis because it reflects not only motility but also DNA integrity [9, 10]. In artificial reproduction practice, sperm morphology evaluation is closely related to sperm morphometric which has been directly linked to male fertility, fertilization success rate, early embryonic development, and pregnancy rate [11]. The sperm's abnormal morphology is believed to be often multifactorial and caused by both genetic and extrinsic [12, 13]

In our study, we demonstrate that exposing frozen-thawed Pasundan Cattle sperm to extreme acidic or alkaline media for a short period increases the number of sperm with abnormal morphology, particularly in the head area. Furthermore, the morphometric characteristics of sperm reveal a significant reduction in sperm surface area, indicating a strong correlation between sperm head abnormal morphology and the reduction in sperm surface area in frozen-thawed Pasundan Cattle sperm incubated in extremely acidic or alkali media for a short period. The results are consistent with previous research that reported a short exposure of frozen-thawed sperm in different incubation media which can significantly increase sperm with abnormal morphology [8]. The reduction of sperm surface area in bovine may be manifested in sperm with head abnormal morphology [14] that is associated with chromatin structure [15, 16] or DNA fragmentation [17, 18].

Naturally, sperm is very sensitive to acidic and alkali extreme environments. In such conditions, sperm will die within 10 minutes at pH 4 and 11. Furthermore, pH 4 is a natural analog of vaginal pH and functions as a naturally spermicidal or microbicidal agent [19]. Furthermore, previous research found that sperm is susceptible to acid denaturation or heat, so exposing frozen-thawed sperm to extreme acidic media for a short period may cause slight shrinkage, resulting in a reduction in sperm surface area. Incubation pH 4 media-induced sperm DNA fragmentation in human sperm [20], as a result, it increases abnormal sperm morphology. At this point we can say that the acidity of media would impact the sperm surface area, suggested is it's related to the change of sperm membrane as the response of different media or environment.

All in all, the results of this study indicate that media incubation with both extreme acid and alkaline would increase the number of sperm with abnormalities in the head area, as well as morphometric. Since sperm heads contain DNA and membrane which are responsible for the fertilization process, the change of both morphology and morphometric of sperm may have a detrimental effect on sperm fertilizing ability.

AUTHORS' CONTRIBUTIONS

RW, DNP, SP, HM, CS, and AB all contributed to the design, experimented, analyzed the data, and

interpreted the results. Furthermore, RW and NMDH were responsible for sample collection and statistical analysis. RW and SP were writing and review the manuscript and approved by all the authors.

ACKNOWLEDGMENTS

This study was funded in share by Doctoral Research Grant from the Indonesian Ministry of Research, Technology, and Higher Education for the fiscal year 2021, contract number 1950/IT3.L1/PN/2021, and a full SEARCA Ph.D. scholarship for Rini Widyastuti.

REFERENCES

[1] Said S, Putra WPB, Anwar S, Agung PP, Yuhani H. Phenotypic, morphometric characterization and population structure of Pasundan cattle at West Java, Indonesia. Biodiversitas. 2017;18(4):1638–45.

[2] Arifin J, Bandiati Komar S, Indrijani H, Rivanda Daud A, Kuswaryan S. The Gene Distribution, Population Equilibrium, Effective Population Size of Pasundan Cattle in Village Breeding Centre at the Southern Part of West Java, Indonesia. In: The UGM Annual Scientific Conference Life Sciences 2016. 2019. p. 216–26.

[3] Santoso S, Herdis H, Arifiantini RI, Gunawan A, Sumantri C. Characteristics and Potential Production of Frozen Semen of Pasundan Bull. Trop Anim Sci J. 2021;44(1):24–31.

[4] Krzyzosiak J, Evenson D, Pitt C, Jost L, Molan P, Vishwanath R. Changes in susceptibility of bovine sperm to in situ DNA denaturation during prolonged incubation at ambient temperature under conditions of exposure to reactive oxygen species and nuclease inhibitor. Reproduction, Fertility and Development.2000; 12(6) 251 – 61.

[5] Chen X, Yue Y, He Y, Zhu H, Hao H, Zhao X, et al. Identification and characterization of genes differentially expressed in X and Y sperm using suppression subtractive hybridization and cDNA microarray. Mol Reprod Dev. 2014;81(10):908–17.

[6] Hwang J, Mannowetz N, Zhang Y, Everley R, Gygi S, Bewersdorf J, et al. Dual Sensing of Physiologic pH and Calcium by EFCAB9 Regulates Sperm Motility. Cell. 2019;177(6):1480-94.e19.

[7] Zhou J, Chen L, Li J, Li H, Hong Z, Xie M, et al. The Semen pH Affects Sperm Motility and Capacitation. PLoS One. 2015;10(7:e0132974):1–15.

[8] Widyastuti R, Maheshwari H, Sumantri C, Pristihadi D, Haq N, Wahyudin, et al. Deleterious effect of short-time incubation at extreme ph to the morphology of frozen-thawed pasundan bull sperm. Vet Pract. 2020;21(2):269–71.

[9] Maroto-Morales A, García-Álvarez O, Ramón M, Martínez-Pastor F, Fernández-Santos M, Soler A, et al. Current status and potential of morphometric sperm analysis. Asian J Androl. 2016;18(6):863–70.

[10] López G, Lafuente R, Checa MA, Carreras R, Brassesco M. Diagnostic value of sperm DNA fragmentation and sperm high-magnification for predicting outcome of assisted reproduction treatment. *Asian J Androl*. 2013;15(6):790-4.

[11] Gadea J. Sperm factors related to *in vitro* and in vivo porcine fertility. Theriogenology. 2005;63(2 SPEC. ISS.):431–44.

[12] Ray PF, Toure A, Metzler-Guillemain C, Mitchell MJ, Arnoult C, Genetic abnormalities leading to qualitative defects of sperm morphology or function.Clin Genet 2017; 91: 217–32.

[13] Oehninger S, Thinus F. Kruger TF. Sperm morphology and its disorders in the context of infertility. F&S Reviews. 2021;2(1): 75-92.

[14] Gravance CG, Casey ME, Casey PJ. Pre-freeze bull sperm head morphometry related to post-thaw fertility. Anim Reprod Sci. 2009;114(1–3):81–8.

[15] Zini A, Phillips S, Courchesne A, Boman JM, Baazeem A, Bissonnette F, et al. Sperm head morphology is related to high deoxyribonucleic acid stainability assessed by sperm chromatin structure assay. Fertil Steril [Internet]. 2009;91(6):2495–500.

[16] Czubaszek M, Andraszek K, Banaszewska D, Walczak-Jędrzejowska R.The effect of the staining technique on morphological and morphometric parameters of boar sperm. PLOS ONE. 2019; 14(3): e0214243.

[17] Dariš B, Goropevnek A, Hojnik N, Vlaisavljević V. Sperm morphological abnormalities as indicators of DNA fragmentation and fertilization in ICSI. Arch Gynecol Obstet. 2010;281(2):363–7.

[18] Enciso M, Cisale H, Johnston SD, Sarasa J, Fernández JL, Gosálvez J. Major morphological sperm abnormalities in the bull are related to

sperm DNA damage. Theriogenology. 2011;76(1):23–32

[19] Olmsted SS, Dubin NH, Cone RA, Moench TR. The rate at which human sperm are immobilized and killed by mild acidity. Fertil Steril. 2000;73(4):687–93

[20] Santiso R, Tamayo M, Gosálvez J, Johnston S, Mariño A, Fernández C, et al. DNA fragmentation dynamics allows the assessment of cryptic sperm damage in human: Evaluation of exposure to ionizing radiation, hyperthermia, acidic pH and nitric oxide. Mutat Res - Fundam Mol Mech Mutagen. 2012;734(1–2):41-9