Protamine-2 protein abundance in Ongole Grade mature spermatozoa is unrelated to semen quality and DNA fragmentation

Berlin Pandapotan Pardede[1*], *Wiwied* Sawitri[2], *Anny* Amaliya[2], *Muhammad* Agil[3], *Iman* Supriatna[3], *Ni Wayan Kurniani* Karja[3], and *Cece* Sumantri[4]

[1]Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia
[2]Singosari Artificial Insemination Center, Singosari, Malang, East Java, Indonesia.
[3]Division of Reproduction and Obstetrics, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, Indonesia
[4]Department of Animal Production and Technology, Faculty of Animal Science, IPB University, Bogor, West Java, Indonesia.

Abstract. This study aimed to analyze the abundance of protein *protamine-2* in mature spermatozoa of Ongole Grade bulls and its relation to semen quality and DNA fragmentation. A total of 120 frozen semen straws from six Ongole Grade bulls aged four to five years were used in this study. The bulls were then divided into two groups, group A (post-thawing motility >45%) and group B (post-thawing motility <45%). The semen quality parameters used in this study included progressive motility, viability, plasma membrane integrity, sperm head defects, and DNA fragmentation. Progressive motility was analyzed using computer-assisted semen analysis. Sperm viability was evaluated using eosin-nigrosine staining. The HOS test was used to assess the integrity of the plasma membrane of spermatozoa. Sperm head defects were analyzed using carbolfuchsin-eosin staining. Sperm DNA fragmentation was analyzed using acridine orange staining. The *protamine-2* protein abundance was analyzed using the enzyme immunoassay method. Although Group A had considerably better semen quality ($P<0.05$) than Group B, it met the artificial insemination criteria. The *protamine-2* protein abundance tended to be higher ($P>0.05$) in group B than in group A. The *protamine-2* protein abundance in Ongole Grade bulls had no relation to semen quality and DNA fragmentation.

1 Introduction

Ongole Grade cattle have been classified as native Indonesian cattle according to the Indonesian Minister of Agriculture Decree No. 2907/KPTS/OT.140/6/2011. Ongole Grade cattle are indigenous Indonesian cattle distinguished by superior reproductive performance and rapid development traits relative to other local breeds [1]. Moreover, a

* Corresponding author: berlinpardede@gmail.com

notable benefit of these native cattle is their tolerance to tropical conditions, particularly tropical illnesses and parasites, and their capacity to flourish under rigorous management and restricted nutrition [2]. One initiative to augment the population of Ongole-grade cattle is the application of artificial insemination with frozen semen. The cryopreserved semen must possess high-quality spermatozoa for optimum fertilization. Santolaria *et al.* [3] showed that spermatozoa quality in cryopreserved semen is critical to fertilization success in artificial insemination programs.

High-quality spermatozoa must be viable, increasingly motile, possess intact plasma membranes, exhibit normal morphology, and have robust DNA integrity [4]. Viable and motile spermatozoa must traverse the cervix and penetrate the cumulus oophores and zona pellucida for fertilization [5]. Tanga *et al.* [4] indicated that spermatozoa require proper morphology, viability, motility, and intact plasma membranes to fertilize oocytes successfully. Saacke [6] asserted that a significant prevalence of defective sperm head morphology would diminish fertilization success. The atypical shape of the spermatozoa head is attributable to hereditary causes, including heightened DNA fragmentation [7]. The significant fragmentation of DNA impedes the embryonic process and contributes to reduced fertility [8]. Numerous variables contributing to DNA fragmentation have been extensively documented, including environmental influences, genetic diseases during spermiogenesis, and chromatin structural abnormalities [9]. Simon *et al.* [8] indicated that reduced protamine levels were associated with increased DNA fragmentation.

There are two main variants of protamine: *protamine-1*, which is the most dominant variant in various species, and *protamine-2*, which has only been reported in a few species and is still unclear regarding its role in normal spermatozoa function [10]. The decrease in fertility in humans has been reported due to the deficiency of *protamine-2* in spermatozoa. Suganuma *et al.* [11] also reported increased DNA fragmentation and decreased semen quality in mice due to impaired *protamine-2* gene expression. Maier *et al.* [12] stated that *protamine-2* was not found in bovine spermatozoa due to gene mutations. A recent report noted that *protamine-2* was found in spermatozoa of Frieswal crossbred bulls, although it did not have a specific role in semen quality [13].

In contrast, Lv *et al.* [14] found that *protamine-2* is required for motility and plasma membrane function in Yanbian Yellow bulls. Nevertheless, Indonesian bulls, especially Ongole Grade bulls, have never been reported. As a result, this study aimed to examine the amount of the protein *protamine-2* in mature spermatozoa from Ongole Grade bulls and its relationship to semen quality and DNA fragmentation.

2 Materials and methods

2.1 Experimental samples

The material utilized in this research was cryopreserved semen generated at the Singosari Artificial Insemination Center, Malang, East Java. They were created by the operating requirements of the Singosari AI Center, namely SNI ISO 9001:2015 No. G.01-ID0139-VIII-2019, under a veterinarian's supervision, considers all animal welfare principles. The frozen semen utilized in the study was sourced from Ongole Grade bulls that had attained sexual maturity and were in their productive period, aged 4 to 5 years. The bulls were

subsequently categorized into two groups: group A (post-thawing motility >45%) and group B (post-thawing motility <45%). One hundred twenty frozen semen straws were thawed in a water bath at 37°C for 30 seconds to assess semen quality and the amount of *protamine-2* protein.

2.2 Semen quality analysis

The parameters analyzed for semen quality in this study encompassed progressive motility, viability, plasma membrane integrity, spermatozoa head shape, and DNA fragmentation. The increasing motility of spermatozoa was assessed using computer-assisted semen analysis as per Pardede *et al.* [5]. Ten microliters of the thawed semen sample were placed on a glass slide and covered with a coverslip. Two hundred to seven hundred fifty cells in four fields were assessed utilizing the Sperm Vision ® CASA System with default settings for bovine spermatozoa. The vitality of spermatozoa was evaluated using an eosin-nigrosine staining technique described by Rodríguez-Martínez [15]. A total of 10 µl of thawed semen was placed on a glass slide and subsequently homogenized using eosin-nigrosine staining. The smear was also prepared from the mixture and dried on a heating table. Two hundred living and deceased spermatozoa cells were examined and enumerated under a microscope at a magnification of 40X. Viable spermatozoa remain unstained (transparent), whereas nonviable spermatozoa exhibit staining (red).

The integrity of the spermatozoa plasma membrane was assessed using the HOS test method described by Pardede *et al.* [16]. A total of 20 µl of thawed semen was added to a microtube containing 300 µl of HOS solution. The solution was incubated in a water bath at 37°C for 30 minutes. One drop of the solution combination was placed on the object glass and then covered with a coverslip. Two hundred spermatozoa were seen and enumerated under a microscope at a magnification of 40X. Spermatozoa exhibiting intact plasma membrane integrity will demonstrate a coiled tail response. The morphology of spermatozoa heads was examined using the carbolfuchsin-eosin staining technique described by Pardede *et al.* [17]. A total of 5-10 µl of thawed semen was applied on a glass slide and subsequently fixed using a Bunsen burner. Subsequently, it was immersed in pure alcohol for 3-4 minutes and then dried. The desiccated smear was washed in a 2% chloramine solution for approximately 2 minutes to eliminate mucus. The smear preparations were rinsed in distilled water and 95% alcohol and then stained in a carbolfuchsin-eosin solution for approximately 6 minutes. The smear was thereafter rinsed with running water and dried. Head morphological anomalies were examined and quantified in 500 spermatozoa cells using a microscope at 40X magnification.

The Acridine Orange (AO) staining technique assessed spermatozoa DNA fragmentation [18]. Five to ten microliters of thawed semen were applied on a glass slide and then fixed with Carnoy's solution for two hours. Following a 5-minute incubation in a dark environment, the smear was treated with AO reagent and subsequently washed with distilled water. The smear was subsequently coated and analyzed with a fluorescence microscope at an excitation wavelength of 450-490 nm. Five hundred cells were examined and enumerated; spermatozoa exhibiting standard DNA integrity emitted green fluorescence, but cells experiencing DNA fragmentation displayed yellow-orange to red fluorescence.

2.3 Analysis of *protamine-2* protein abundance

The amount of *protamine-2* was assessed using the enzyme-linked immunosorbent assay (ELISA) method. Phosphate-buffered saline (PBS) solution was utilized to wash 100 µl of thawed semen, which had been centrifuged at 3000 rpm for 15 minutes. The procedure is conducted two times. The analysis of *protamine-2* protein abundance was performed using the bovine *protamine-2* kit from MyBioSource.com. The analytical method is initiated by adding the standard diluent and sample to the designated wells. Ten microliters were introduced into the sample well, sealed, and incubated for 45 minutes at 37°C. Each well was aspirated and washed with buffer four times, resulting in five washes. Subsequently, introduce the HRP-conjugated detection antibody to each well, cover the wells, and incubate for 30 minutes at 37°C. Introduce chromogen solutions A and B to each healthy sample five times following aspiration and plate washing. Incubate the plate for 15 minutes at 37°C in the dark. Subsequently, the stop solution was introduced to each well, and the optical density (OD) was measured at 450 nm.

2.4 Statistical analysis

Data comparing semen quality and protein abundance of *protamine-2* in the two groups were analyzed using the student T-test. The Spearman correlation test and simple linear regression investigated the connection between *protamine-2* protein quantity and semen quality indicators. SPSS version 25.0 was used for all data analysis in this study (IBM, Armonk, NY, USA). The data is provided as a mean with a mean standard error (SEM).

3 Results and discussion

In comparison to group B, the progressive motility of spermatozoa in group A was considerably greater ($P<0.05$) (Table 1; Figure 1). These results were then used as the basis for grouping Ongole Grade bulls to determine semen quality and protein abundance of *protamine-2*. Progressive motility is the percentage of spermatozoa that move forward. Spermatozoa motility is the most crucial characteristic related to fertilizing and is one of the quality controls for insemination doses before and after thawing [19]. Hirose *et al.* [5] indicated that only spermatozoa exhibiting progressive motility can traverse the cervix and reach the cumulus oophores and zona pellucida of the ovum, a prerequisite for fertilization. Both groups in the study showed progressive motility values that were still in the normal range, which still met the requirements of SNI 48691:2017 for post-thawing frozen semen with progressive motility of at least 40% [20]. Pardede *et al.* [17] reported that spermatozoa with progressive motility of more than 49% could produce a more than 70% conception rate.

Viability is an essential measure of sperm quality and one of the prerequisites for spermatozoa to fertilize oocytes [21]. The viability data revealed a substantial difference between the two motility groups, with group A being significantly greater ($P<0.05$) than group B (Table 1). According to Reveco *et al.* [22], the proportion of spermatozoa viability in frozen sperm considered good for AI was between 64 and 80 percent. Based on this, the results of the percentage of viability in both groups were in good condition for

artificial insemination. The results showed that the plasma membrane integrity in group A was significantly higher (P<0.05) compared to group B (Table 1). The India Agri Ministry [23] stated that at least the plasma membrane integrity of spermatozoa in frozen semen was 40%. The integrity of the plasma membrane is critical in enhancing spermatozoa performance since only sperm cells with intact membranes may thrive and undergo several complicated adaptations in the female reproductive tract before fertilizing eggs [16]. The percentage of plasma membrane integrity in both groups was still within the normal range. The difference in semen quality in each of these groups can be caused by various factors, including cryopreservation factors that impact semen quality, which vary from individual to individual. The cryopreservation process will cause a substantial decrease in the intracellular antioxidants of spermatozoa and make them more susceptible to damage due to oxidative stress (ROS) reactions [16].

Table 1. Characteristics of frozen semen from Ongole Grade bulls in two different motility groups.

Variables	Sperm motility groups	
	A	B
Progressive motility (%)	49.86±0.77[a]	44.29±0.51[b]
Sperm viability (%)	74.80±0.95[a]	68.47±0.68[b]
Plasma membrane integrity (%)	74.80±0.89[a]	68.57±0.62[b]
Head defects (%)	1.91±0.12[a]	4.02±0.13[b]
DNA fragmentation (%)	0.95±0.09[a]	3.13±0.20[b]

Means in identical rows with various superscripts vary significantly (P<0.05).

Several complex cellular events generate the usual morphological structure of spermatozoa, and any modifications during these steps will affect structural abnormalities [17]. Head anomalies are primary abnormalities during spermatozoa formation in the testes and negatively impact fertility. The findings revealed that the proportion of spermatozoa head abnormalities in group A was substantially lower (P<0.05) than in group B (Table 1; Figure 1). The average spermatozoa abnormality in cows was 20%, with overall anomalies ranging from 8% to 12% in bulls with high fertility [24]. Based on this, the morphological abnormalities of spermatozoa in this study were still in excellent and average condition. Saacke [6] reported that primary abnormalities could have significant consequences for males because they occur continuously, can be passed on to offspring, and interfere with male fertility.

DNA fragmentation will affect the inability of the embryogenesis phase and is one of the causes of reduced fertility [25]. The results of spermatozoa DNA fragmentation analysis using AO staining (Figure 2) showed that DNA fragmentation in group A was substantially lower (P<0.05) than in group B (Table 1; Figure 1). DNA fragmentation in the sperm of less than 15% is considered normal, while 15% to 25% reduces fertility, and more than 25% is categorized as sterile males, according to D'Occhio *et al.* [26]. Based on this, this study's DNA damage percentage was still within the normal range. Various variables, including changes in the structure of specific nucleic proteins, such as protamine, might induce DNA fragmentation alterations [27]. At the spermatozoa maturation stage, the primary protein transition process in the nucleus, initially dominated by histones, became protamine-mediated by transition proteins [28]. The

expression ratio of these two proteins determines chromatin condensation in carrying out its function of maintaining DNA integrity [27].

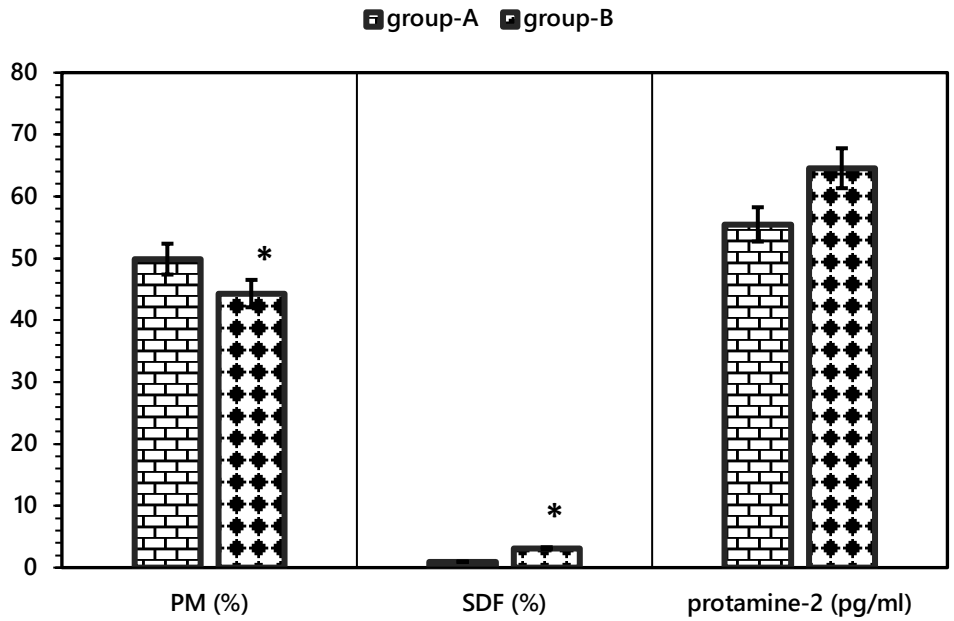

Fig. 1. Graph of progressive spermatozoa motility values, DNA fragmentation, and protein abundance of *protamine-2* in two motility groups.

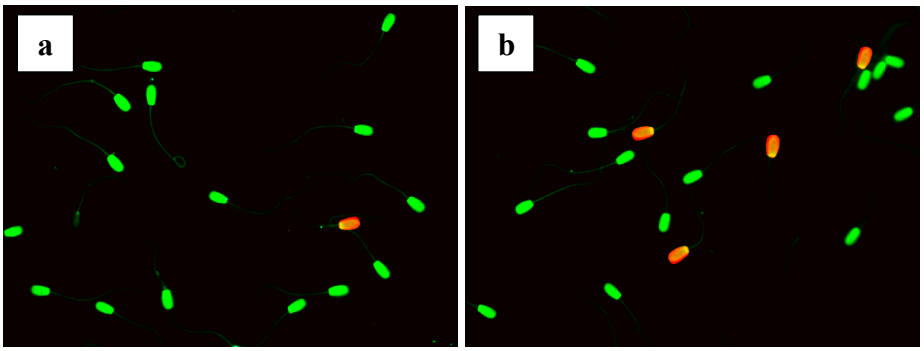

Fig. 2. DNA fragmentation analysis using AO staining in groups A (a) and B (b): Spermatozoa with normal DNA content will be dyed with green fluorescence, whereas those with DNA damage will be dyed with orange to yellow fluorescence.

Protamine-2 in this study was detectable and measurable overall (Figure 1). This result is different from Maier *et al.* [12], who stated that *protamine-2* was not found in mature spermatozoa due to mutations in the RNA level in the testes. The protein abundance of *protamine-2* in the research did not indicate any significant disparities (P>0.05) between the two different motility groups (Figure 1), where the protein abundance of *protamine-2* in group A (55.47±6.99 pg/ml) was relatively lower than in group B (64.55±5.45 pg/ml). The study found no association between *protamine-2* protein quantity and semen quality

or spermatozoa DNA fragmentation (Figure 3; Figure 4; Table 2). This finding differs from Lv *et al.* [14], who found that *protamine-2* in Yanbian Yellow bulls has an essential effect on semen quality, including motility and spermatozoa plasma membrane. In addition, this is also different from that reported in mice, that DNA fragmentation occurs in spermatozoa with *protamine-2* deficiency.

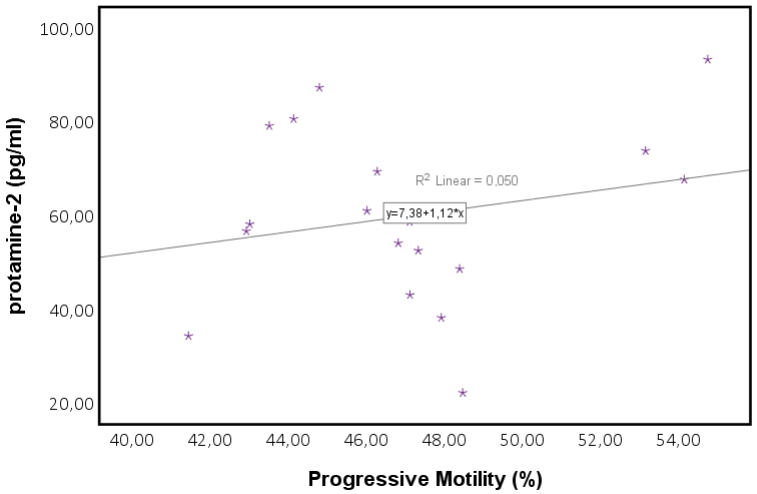

Fig. 3. Linear regression graph between protein abundance of *protamine-2* and progressive motility of spermatozoa (r=-0.003).

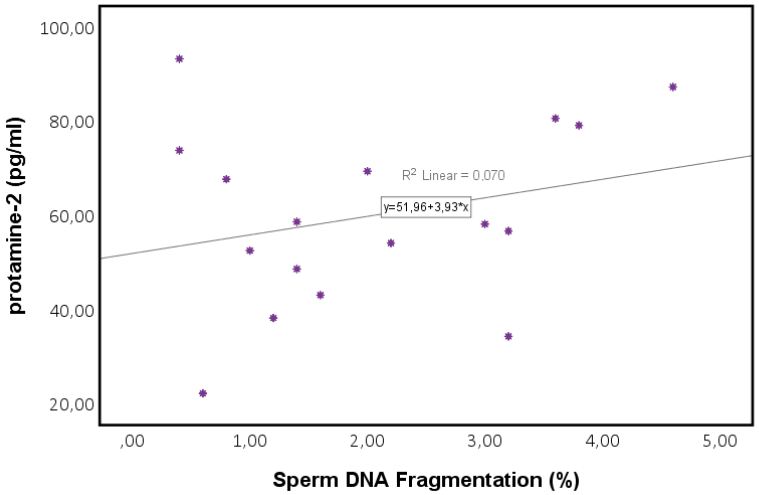

Fig. 4. Linear regression graph between protein abundance of *protamine-2* and spermatozoa DNA fragmentation (r=0.180).

However, several additional research on bovine spermatozoa back up the findings of the present study. Ganguly *et al.* [13] found that *protamine-2* in Frieswal bull's spermatozoa did not affect semen quality. Unlike in humans and mice, where the ideal ratio of *protamine-1* and *2* should be 1:1 [10], in bulls, it has been reported that although *protamine-2* is found in spermatozoa, it does not have a specific role in normal

spermatozoa function [29]. *Protamine-1* is the most prevalent protamine variation, according to Moghbelinejad *et al.* [30], and it plays an essential role in proper spermatozoa function, including its link to the degree of DNA fragmentation. Protamine, conversely, is the primary protein found in the core of the spermatozoa and is required for parental chromosomal compaction [28]. This DNA-protamine attachment will result in a spermatozoa nucleus that is more compact and hydrodynamic [27]. Spermatozoa with a hydrodynamic core are essential in fertilization, as they can swim quicker and fertilize oocytes [10].

Table 2. Correlation between protein abundance of *protamine-2* and semen quality parameters

Parameter	P2	PM	SV	MI	HD	SDF
P2	1	-0.003	0.070	0.087	0.240	0.180
PM (%)		1	0,867**	0,882**	-0,877**	-0.907**
SV (%)			1	0,963**	-0,708**	-0.754**
PMI (%)				1	-0,781**	-0.806**
HD (%)					1	-0.944**
SDF (%)						1

**Correlation is significant at the 0.01 level; P2: *protamine-2*; PM: progressive motility; SV: sperm viability; PMI: plasma membrane integrity; HD: head defect; SDF: sperm DNA fragmentation.

4 Conclusions

The *protamine-2* protein abundance in Ongole Grade bulls had no relation to semen quality and DNA fragmentation.

Acknowledgment

The author(s) declare that no financial support was received for this article's research, authorship, and/or publication.

References

1. E. Romjali, Local beef cattle breeding program in Indonesia, Wartazoa. **28**, 190-210 (2018). http://dx.doi.org/10.14334/wartazoa.v28i4.1813
2. G. Sitanggang, Environmental effects and repeatability estimates on semen quality in Ongole Grade bulls. JIPI. **23**, 88-92 (2018). http://dx.doi.org/10.18343/jipi.23.2.88
3. P. Santolaria, J.P. Rickard, R. Pérez-Pe, Understanding sperm quality for improved reproductive performance. Biology. **12**, 980 (2023). https://doi.org/10.3390/biology12070980
4. B.M. Tanga, A.Y. Qamar, S. Raza, S. Bang, X. Fang, K. Yoon, J. Cho, Semen evaluation: methodological advancements in sperm quality-specific fertility assessment - A review. Animal bioscience. **34**, 1253–1270 (2021). https://doi.org/10.5713/ab.21.0072
5. M. Hirose, A. Honda, H. Fulka, M. Tamura-Nakano, S. Matoba, T. Tomishima, K. Mochida, A. Hasega, K. Nagashima, K. Inoue, M. Ohtsuka, T. Baba, R. Yanagimachi, A. Ogura, Acrosin is essential for sperm penetration through the zona pellucida in

hamsters. Proc Natl Acad Sci U S A. **117**, 2513-2518 (2020). https://doi.org/10.1073/pnas.1917595117

6. R.G. Saacke. Sperm morphology: its relevance to compensable and uncompensable traits in semen. Theriogenology. **70**, 473-478 (2018). https://doi.org/10.1016/j.theriogenology.2008.04.012
7. M. Kleshchev, A. Osadchuk, L. Osadchuk, Impaired semen quality, an increase of sperm morphological defects and DNA fragmentation associated with environmental pollution in urban population of young men from Western Siberia, Russia. PLOS ONE. **16**, e0258900 (2021). https://doi.org/10.1371/journal.pone.0258900
8. L. Simon, J. Castillo, R. Oliva, S.E.M. Lewis, Relationships between human sperm protamines, DNA damage, and assisted reproduction outcomes. Reprod Biomed Online. **23**, 724-34 (2011). https://doi.org/10.1016/j.rbmo.2011.08.010
9. T. Wang, H. Gao, W. Li, C. Liu, Essential role of histone replacement and modifications in male fertility. Front Genet. **10**, 962 (2019). https://doi.org/10.3389/fgene.2019.00962
10. R. Olivia, Protamines and male infertility. Hum Reprod Update. **12**, 417-35 (2006). https://doi.org/10.1093/humupd/dml009
11. R. Suganuma, R. Yanagimachi, M.L. Meistrich, Decline in fertility of mouse sperm with abnormal chromatin during epididymal passage as revealed by ICSI. Hum Reprod. **20**, 3101–3108 (2005). https://doi.org/10.1093/humrep/dei169
12. W.M. Maier, G. Nussbaum, L. Domenjoud, U. Klemm, W. Engel, The lack of protamine 2 (PT 2) in boar and bull spermatozoa is due to mutations within P2 gene. Nucleic Acids Res. **18**, 1249–1254 (1990). https://doi.org/10.1093/nar/18.5.1249
13. I. Ganguly, G.K. Gaur, S. Kumar, D.K. Mandal, M. Kumar, U. Singh, S. Kumar, A. Sharma, Differential expression of protamine 1 and 2 genes in mature spermatozoa of normal and motility impaired semen producing crossbred Frieswal (HF x Sahiwal) bulls. Res Vet Sci. **94**, 245-62 (2013). https://doi.org/10.1016/j.rvsc.2012.09.001
14. Y.Q. Lv, X. Chen, D. Xu, X.T. Luo, M.M. Cheng, Y.Y. Zhang, X.L. Qu, Y. Jin Y, Effects of crocin on frozen-thawed sperm apoptosis, protamine expression and membrane lipid oxidation in Yanbian yellow cattle. Reprod Domest Anim. **55**, 1011-1020 (2020). https://doi.org/10.1111/rda.13744
15. H. Rodríguez-Martínez, Evaluation of frozen semen: traditional and new approaches. In: Topics in Bull Fertility (Chenoweth PJ, International Veterinary Information Service, Ithaca, New York, USA, 2000).
16. B.P. Pardede, A. Kusumawati, M. Pangestu, B. Purwantara, Bovine sperm HSP-70 molecules: a potential cryo-tolerance marker associated with semen quality and fertility rate. Front Vet Sci. **10**, 1167594 (2023). https://doi.org/10.3389/fvets.2023.1167594
17. B.P. Pardede, N.W.K. Karja, S. Said, E.M. Kaiin, M. Agil, C. Sumantri, B. Purwantara, I. Supriatna, Bovine nucleoprotein transitions genes and protein abundance as valuable markers of sperm motility and the correlation with fertility. Theriogenology. **215**, 86-94 (2024). https://doi.org/10.1016/j.theriogenology.2023.11.015
18. A. Kusumawati, F.A. Satrio, R. Indriastuti, Z.N.A. Rosyada, B.P. Pardede, M. Agil, B. Purwantara, Sperm head morphology alterations associated with chromatin instability and lack of protamine abundance in frozen-thawed sperm of indonesian local bulls. Animals. **13**, 2433 (2023). https://doi.org/10.3390/ani13152433
19. R. Puglisi, A. Pozzi, L. Foglio, M. Spano, P. Eleuteri, M.G. Grollino, G. Bongioni, A. Galli, The usefulness of combining traditional sperm assessments with in vitro heterospermic insemination to identify bulls of low fertility as estimated in vivo. Anim Reprod Sci. **132**, 17-28 (2012). https://doi.org/10.1016/j.anireprosci.2012.04.006

20. BSN (Badan Standarisasi Nasional), SNI (Standar Nasional Indonesia) Semen Beku - Bagian 1: Sapi SNI 4869.1-2017 (Badan Standarisasi Nasional, Jakarta, Indonesia, 2017).
21. J.M. Morrell, H. Rodriguez-Martinez, Biomimetic techniques for improving sperm quality in animal breeding: a review. J Androl. **1**, 1-9 (2009).
22. A.R. Reveco, J.L. Hernández, P. Aros. Long-term storing frozen semen at −196 °C does not affect the post-thaw sperm quality of bull semen in cryopreservation in eukaryotes. IntechOpen. p91101 (2016).
23. India Agriculture Ministry, Compendium of minimum standards of protocol and standard operating procedures for bovine breeding (Department of Animal Husbandry, Dairying, and Fisheries, India, 2014).
24. G.K. Patel, N. Haque, M. Madhavatar, A.K. Chaudhari, D.K. Patel, N. Bhalakiya, N. Jamnesha, P. Patel, R. Kumar, Artificial insemination: A tool to improve livestock productivity. J Pharm and Phytoch. **1**, 307-313 (2017).
25. A. Khezri, B. Narud, E. Stenseth, A. Johannisson, F.D. Myromslien, A.H. Gaustad, R.C. Wilson, R. Lyle, J.M. Morrell, E. Kommisrud, R. Ahmad, DNA methylation patterns vary in boar sperm cells with different levels of DNA fragmentation. BMC Genomics. **20**, 897 (2019). https://doi.org/10.1186/s12864-019-6307-8
26. J.M. D'Occhio, K.J. Hengstberger, D. Tutt, R.G. Holroyd, G. Fordyce, G.B. Boe-Hansen, S.D. Johnston, Sperm chromatin in beef bulls in tropical environments. Theriogenology. **79**, 946-52 (2013). https://doi.org/10.1016/j.theriogenology.2013.01.012
27. A. Champroux, J. Torres-Carreira, P. Gharagozloo, J.R. Drevet, A. Kocer, Mammalian sperm nuclear organization: resiliencies and vulnerabilities. Basic Clin Androl. **26**, 17 (2016). https://doi.org/10.1186/s12610-016-0044-5
28. L. Moritz, S.B. Schon, M. Rabbani, Y. Sheng, R. Agrawal, J. Glass-Klaiber, C. Sultan, J.M. Camarillo, J. Clements, M.R. Baldwin, A.G. Diehl, A.P. Boyle, P.J. O'Brien, K. Ragunathan, Y.C. Hu, N.L. Kelleher, J. Nandakumar, J.Z. Li, K.E. Orwig, S. Redding, S.S. Hammoud, Sperm chromatin structure and reproductive fitness are altered by substitution of a single amino acid in mouse protamine 1. Nat Struct Mol Biol. **30**, 1077-1091 (2023). https://doi.org/10.1038/s41594-023-01033-4
29. T.R.S. Hamilton, R. Simoes, C.M. Mendes, M.D. Goissis, E. Nakajima, E.A.L. Martins, J.A. Visintin, M.E.O.A. Assumpcao, Detection of protamine 2 in bovine spermatozoa and testicles. Andrology. **7**, 373-381 (2019). https://doi.org/10.1111/andr.12610
30. S. Moghbelinejad, R. Najafipour, A.S. Hashjin, Comparison of protamine 1 to protamine 2 mRNA ratio and ybx2 gene mRNA content in testicular tissue of fertile and azoospermic men. Int J Fertil Steril. **9**, 338-45 (2015). https://doi.org/10.22074/ijfs.2015.4549