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Evaluation Deoxyribonucleic acid (DNA) fragmentation of local Indonesian cattle frozen sperm using Halomax® method

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Abstract. *Prabowo TA, Bintara S, Yusiati LM, Sitaresmi PI, Widayati. 2023. Evaluation Deoxyribonucleic acid (DNA) fragmentation of local Indonesian cattle frozen sperm using Halomax® method. Biodiversitas 24: 2225-2230.* Deoxyribonucleic acid (DNA) is a molecular chain containing genetic material that determines the quality of each individual produced. DNA damage in cattle can affect fertility and miscarriage of embryos. There is no simple research on DNA damage in local Indonesian cattle (*Bos indicus* Linnaeus 1758); therefore, miscarriage and infertility cases in Indonesia are still high, one of which can be attributed to sperm DNA damage. This study aimed to evaluate DNA damage in the frozen semen of local Indonesian cattle and suggested SDF (Sperm DNA Fragmentation) inclusion screening as the main requirement for frozen semen. The materials used in this study were 54 straws of frozen semen from nine males from three different types of local cattle, including Bali, Madura, and Aceh. Each type consisted of three bulls, each with six replicates. Sperm-*Bos*-Halomax was used to examine sperm DNA damage. Data were analyzed using one-way analysis of variance (ANOVA). Aceh cattle had the highest post average compared to Bali and Madura at 63.33±2.43%. Abnormality of Balinese cattle had the lowest average compared to Aceh and Madura at 8.83±1.04%. Evaluation of sperm DNA damage in local cattle using the Sperm-*Bos*-Halomax® method showed that the highest average DNA damage was 1.72±0.62% in Aceh, and the lowest was 1.67±0.66% in Madura cattle. The sperm DNA damage in Bali cattle was 1.67±0.70%. The sperm DNA quality in the frozen semen of the local cattle in this study was good, but it is still necessary to determine SDF as the main standard in frozen semen.

Keywords: Aceh cattle, artificial insemination, Bali cattle, DNA fragmentation, Madura cattle, sperm, sperm-Bos-Halomax®

INTRODUCTION

Indonesia has a variety of potential beef cattle, including Bali (*Bos indicus* Linnaeus 1758), Aceh (*B. indicus* hybrid), and Madura (*B. indicus* hybrid), which have characteristics suited to tropical environments. Many studies have already been conducted on these breeds (Isnaini et al. 2019; Kurniawan et al. 2020; Suyadi et al. 2022). In general, tropical weather conditions negatively affect farm animals. Relatively hotter temperatures and high humidity affect the hemostasis of livestock to be unbalanced for livestock from colder countries such as Europe (Tanga et al. 2021). In addition to the scarcity of desirable feed, the issue of small farm animal ownership by local farmers (i.e., one farmer retains only six cattle and uses agricultural waste as a source of animal feed) is a major impediment to the stagnation of the cattle industry, particularly in developing nations (Agus and Widi 2018). Artificial Insemination (AI), a reproductive technology using local cattle, has been used as one of the chosen attempts to increase the local cattle reproductive and solve small population problems and environmental conditions (Widyastuti et al. 2021). Infertility has long been a source of the issue and is a concern in many organisms continuously, including farm animals. Though massive research on farm animals is scarce, existing data indicate that cattle's subfertility is increasing. This can negatively impact animal welfare and farm profitability through delayed calving intervals and increased animal culling. Previous studies reported that the first-service pregnancy rate in dairy cattle has decreased from 70% to 40% during the last 30 to 50 years (Tadesse et al. 2022). Male and female factors contribute to infertility, although male-factor infertility accounts for 40-50% (Kumaresan et al. 2020). It was found that the "acceptable grade semen generating ability" of some bulls diminished through time, from grandsire to sire to male progeny (Thippeswamy et al. 2014). The importance of male fertility is magnified in farm animals since sperm from a male is utilized for artificial insemination in thousands of females, and sperm from infertile bulls influences conception, resulting in significant losses for farmers.

Livestock production places significant importance on the ability to determine paternal fertility reliably. Although the performance of sperm has been traditionally explained in terms of cell viability, motility, and morphology, it has been noticed that these aspects are rarely significant predictors of fertility results in terms of the field. The investigation is often accused of female factors because the semen checker was performed beforehand using the traditional approach,

which is no longer reliable in Assisted Reproductive Technologies (ART) protocols (Tanga et al. 2021). Therefore, examining spermatozoa DNA damage is important to confirm fertility (Arif et al. 2020). Even though sperm DNA fragmentation (SDF) has not been evaluated in standard sperm assays, studies indicate that SDF has a major influence on male fertility. Diverse hypotheses have been proposed to explain sperm DNA damage (apoptosis, chromatin remodeling, and oxidative damage). Successful conception occurs only when sperm with intact DNA pollinates eggs during natural mating. Conversely, implementing ART, such as artificial insemination (AI), in almost all reproductive management processes in tropical developing countries has enhanced the possibility of DFS fertilizing oocytes (de Mouzon et al. 2020). SDF can occur during spermatogenesis, freezing, and thawing. Damage can be triggered by factors including: age, testicular infection, lack of protamine elements, hormones, contamination from toxic chemicals and drugs, testicular hyperthermia, apoptosis, and high levels of reactive oxygen species (ROS) (Abdillah et al. 2019). Moreover, SDF damage caused miscarriages or abortions in Brahman cattle at 14.31%, occurring on average at 45 days of gestation (Priyanto et al. 2019). The mortality rate of embryos (using ultrasound examination) from artificial insemination in Yogyakarta reached 80% (Priyanto et al. 2019). This may be due to DNA damage caused by the freezing process or other reasons. SDF causes spontaneous abortion, stillbirth, and inherited diseases (Yan et al. 2022). These conditions lead to a prolonged estrus cycle, which results in great losses for farmers with longer calving intervals or open days. When females are in estrus again, they dispose of the same semen straw by the AI operator, repeating the same condition.

Therefore, it is necessary to evaluate the DNA damage of spermatozoa in frozen semen produced by national insemination centers. Therefore, among various types of local Indonesian cattle produced, the main producers of frozen semen circulated in the field are in a superior condition.

MATERIALS AND METHODS

Study area

This research was conducted at the National Artificial Insemination Center, Singosari, Malang, Indonesia, and the Laboratory of Animal Reproductive Physiology, Faculty of Animal Husbandry, Gadjah Mada University, Yogyakarta, Indonesia.

Procedures

Frozen bull semen (54 straws) from Bali, Madura, and Aceh males were acquired from the Artificial Insemination Center in Singosari, East Java. Cattle are kept in specialized stud breeding pens at 1,200 mdpl with a temperature of 16-22°C. Furthermore, the feed was given in greenery and concentrated at a ratio of 60:40/body weight/day. All chemicals and reagents used in this study were obtained from Sigma-Aldrich (St. Louis, MO). Semen with sperm motility above 70% and a concentration above 1000 million/mL was processed into frozen semen (BSN 2017). The process was carried out based on the Singosari BIB protocol using egg yolk diluents with a glycerol concentration of 8%. It was then packaged in a 0.25 mL mini straw with an insemination dose of 25 million/straw. Frozen semen was stored in a liquid nitrogen container (-196°C) for further testing.

Post thawing motility

Frozen semen testing is done by thawing the frozen semen first. Frozen semen was thawed in water at 37°C for 30 seconds. Semen that has been thawed is put into a microtube and evaluated for post-thawing motility. Observations were made using a microscope (Olympus CH 20) with 100x and 400x magnification at 10 fields of view. In addition, ratings were given in the 0-100% range with a 5% scale.

Abnormality

Frozen semen that has been thawed at 37°C for 30 seconds is then made into thin preparations by dripping 0.05 mL of frozen semen into an object glass and adding 0.05 mL of eosin dye, after which it is homogenized. Then, 0.05 mL of the mixed solution between sperm and eosin was leveled on the object glass. After that, it was observed under a microscope. Finally, observations of spermatozoa abnormality were carried out using a light microscope (Olympus CH 20) with 400 times magnification. Counting the number of abnormalities was performed in 10 different fields of view.

Sperm DNA fragmentation Halomax methods.

The quality of the frozen semen was tested by thawing it in 37°C of water for 30 s. The thawed semen was placed in a microtube, and DNA integrity was evaluated. Semen with a final concentration of 15-20 million cells/mL was placed in Phosphate Buffered Saline (PBS). DNA fragmentation testing using a Halomax kit followed by agarose being thawed in a water bath at 90°C-100°C for 5 min. The temperature of the agarose solution was then lowered to 37°C. Next, 25 µL of frozen semen that had been thawed was mixed with 25 µL of agarose solution into a microtube. The cement and agarose solution mixture was then dripped as much as 25 µL into the glass object covered with a cover glass; these specimens were incubated in a refrigerator for 5 min. Next, the cover glass was slowly lifted and dripped with Lysis Solution (LS) until the agarose was submerged. The mixture was then incubated at room temperature for 5 min. The specimens were then incubated in distilled water for 5 min, followed by 70, 90, and 100% ethanol (within 4 min each), and dried. The specimen was then incubated in distilled water for 5 minutes, dried, stained by incubation in a box containing eosin for 5 minutes, and washed with distilled water in a box for 2 (two) minutes. The specimens were then incubated in a box containing Methylene Blue dye for 5 (five) minutes and washed with distilled water for 2 (two) minutes. Specimens were observed under 400x magnification microscope with a green filter to view the DNA chromatin threads on the head of the sperm (halo). In

addition, the examination was performed on 500 spermatozoa from each sample.

Data analysis

All data were expressed as mean value ± SEM. Data were analyzed using one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Post-thawing motility and abnormality

The results showed that Aceh cattle had the highest post average compared to Bali and Madura cattle at 63.33±2.43%. On the other hand, the abnormality of Balinese cattle had the lowest average compared to Aceh and Madura cattle at 8.83±1.04% (Table 1).

Sperm DNA fragmentation Halomax methods

The result of sperm DNA testing using the Sperm–Bos–Halomax® method is to observe sperm DNA fragmentation (Sperm DNA fragmentation index; SDFi). DNA fragmentation in sperms with DNA damage can be examined from chromatin's release from the cell, forming a glowing *halo*. However, for those with intact DNA, the glowing *halo* does not appear (Figure 1). The evaluation results of the sperm DNA damage in the local cattle with Sperm–*Bos*–Halomax® show that the highest average DNA damage is 1.72±0.62% found in Aceh, and the lowest is 1.67±0.66% found in Madura cattle. At the same time, the DNA damage in Bali cattle was 1.67±0.70% (Table 2). The study's results stated that the livestock type affected the spermatozoa DNA quality ($P<0.05$). This is thought to be due to the geographical location of different environments affecting semen quality.

Discussion

Post-thawing motility and abnormality

Large seasonal oscillations in the reproduction of tropical bulls have been linked to changes in nutrition, habitat, phenotype, and possibly photoperiod (Ratnani et al. 2020). But seasonal changes in herd fertility are rarely caused by bull-related variables. However, bulls from tropical regions also show seasonal fluctuations in semen quality and freezing capability, the severity of which can result in economic losses and varies depending on genotype. Additionally, tropical countries are characterized by maritime geography and are typically composed of numerous small islands that are widely spaced. Thus, the beef cattle industry's ability to deal with this challenge will greatly benefit from well-managed artificial insemination with frozen semen (Ratnani et al. 2020). Therefore, to further enhance sperm quality, a beneficial solution in the form of extra active substances is needed (Bustani and Baiee 2021).

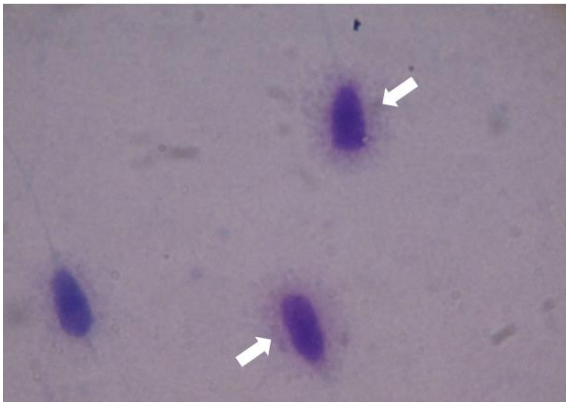

Figure 1. The sperm DNA fragmentation of cattle marked with arrows, as tested using the Sperm–Bos–Halomax ® kit at 40x magnification.

Table 1. Post-thawing motility and spermatozoa abnormality of frozen semen

Types of cattle	Names of cattle	Parameters	
		Post-thawing motility (%)	Abnormalities (%)
Bali	Bali 1	61.67±2.58	8.67±0.82
	Bali 2	62.50±2.74	8.50±1.05
	Bali 3	63.33±2.58	9.33±1.21
	Average	62.50±2.58	8.83±1.04
Madura	Madura 1	62.50±2.74	11.50±1.38
	Madura 2	63.33±2.58	11.00±1.26
	Madura 3	61.67±2.58	13.50±1.05
	Average	62.50±2.57	12.00±1.61
Aceh	Aceh 1	64.17±2.04	13.83±1.33
	Aceh 2	63.33±2.58	16.33±1.21
	Aceh 3	62.50±2.74	15.33±1.51
	Average	63.33±2.43	15.17±1.65

Table 2. Sperm DNA fragmentation in the frozen semen of local cattle

Types of cattle	Names of cattle	Parameters	
		DNA damage (%)	Normal DNA (%)
Bali	Bali 1	1.83±0.75	98.17±0.75
	Bali 2	1.50±0.84	98.05±0.84
	Bali 3	1.67±0.52	98.33±0.52
	Average	1.67±0.70[a]	98.18±0.70[b]
Madura	Madura 1	2.00±0.63	98.00±0.63
	Madura 2	1.33±0.52	98.67±0.52
	Madura 3	1.67±0.82	98.33±0.82
	Average	1.67±0.66[a]	98.33±0.66[b]
Aceh	Aceh 1	1.50±0.55	98.50±0.55
	Aceh 2	2.17±0.75	97.83±0.75
	Aceh 3	1.50±0.55	98.50±0.55
	Average	1.72±0.62	98.28±0.62
Total		12.28±1.53[a]	87.73±1.53[b]

Cryopreservation solves these problems but can greatly extend the time bull spermatozoa must be stored before use. It also allows for long travel distances (Ugur et al. 2019). However, it still presents several challenges during cryopreservation and thawing that significantly degrade semen quality and impair fertility. These include: intracellular and extracellular frost emergence, cold shock, compound toxic effects of cryoprotectants, osmolarity concussion, lipid oxidation concussion, and apoptosis (Peris-Frau et al. 2020). In addition, cryopreservation has been linked to several negative outcomes, including: increased plasma membrane fluidity and permeability, excessive formation of reactive oxygen species (ROS), compromised acrosome integrity (Bollwein and Bittner 2018), reduced mitochondrial membrane potential, and impaired sperm motility. The Bull spermatogenic membranes are highly permeable and contain high polyunsaturated fatty acids (PUFAs). That could make them vulnerable to lipid peroxidation damage caused by oxygen exposure. Because of this, they are susceptible to reactive oxygen species (ROS) invasion, which has been shown to negatively impact sperm motility, sperm activation, and acrosome reaction. This is probably because low sperm viability and axonemal damage result from a substantial drop in intracellular ATP levels (Collodel et al. 2021).

During the freezing process, semen undergoes various temperature and osmotic pressure changes that will affect spermatozoa quality (Table 1). The damage can be understood because spermatozoa experience multiple extreme temperature drops during the freezing process. The first drop occurs during equilibration, from room temperature to 5°C; the spermatozoa experience cold shock. The next drop is during freezing in liquid nitrogen vapor, which is thought to be -130°C. This cold shock causes damage to spermatozoa, especially to the plasma membrane. In addition, the plasma membrane is the outer spermatozoa protector directly affected by environmental changes. Moreover, if damage occurs to the spermatozoa membrane in the head, it will be detected in the abnormality examination. Therefore, many spermatozoa will be colored by the dye given. In addition, if damage occurs in the tail, especially in the midpiece, the mitochondria will be disrupted so that Adenosine Tri Phosphate (ATP) is not produced, and motility will be stopped. Pardede et al. (2022) stated that to produce ATP, the enzyme ATP-transferase is needed; if the membrane in the midpiece is damaged, it is suspected that this enzyme will be lost, and the ability to move is also stopped.

Besides cold shock, the damage can also be caused by semen diluents containing hyperosmotic cryoprotectants. Diluents containing cryoprotectants have an osmotic pressure above 1000 mOsm kg^{-1}, while the osmotic pressure of fresh semen is between 250-350 mOsm/kg (Herbowo et al. 2019). Therefore, freezing can reduce motility between 30% and 60%, causing changes in spermatozoa morphology and also mitochondrial and acrosome damage (Hussain et al. 2019). The freezing technique, extender, and the type and concentration of cryoprotective agent determine the quality of frozen semen. Furthermore, glycerol is widely used as a cryoprotective agent (CPAs) for cryopreservation on buffalo semen (*Bos taurus* Linnaeus 1758) (Correia et al. 2021). Tariq et al. (2020) reported that 6% glycerol added at 37°C provided a better cryoprotection to the motility apparatus and plasma membrane integrity of buffalo sperm. As an intracellular CPA, Glycerol permeates the cells to prevent ice crystal formation. Besides their benefits, some studies reported that glycerol could induce physicochemical alteration. That could lead to plasma-membrane rupture, membrane protein removal, and acrosome damage, which would reduce sperm fertility.

Evaluation of spermatozoa DNA fragmentation

Sperm DNA testing using the Sperm–*Bos*–Halomax® method determined the sperm DNA fragmentation index (SDFi) (Baiee et al. 2018) (Figure 1). The evaluation of sperm DNA damage is important for revealing infertility in males and examining motility and viability (Nugraha et al. 2022). Damage to the sperm's DNA can lead to embryonic death. Sperm DNA damage greatly affects fertilization, preimplantation development, and embryonic development. Sperm DNA damage influences embryonic development and correlates positively with the rate of miscarriage (Priyanto et al. 2019). The higher the level of sperm DNA damage, the higher the miscarriage rate (Serafini et al. 2016). However, until recently, the detection of SDF was not the main requirement for the standardization of frozen semen. Furthermore, the type of livestock affected the quality of spermatozoa DNA; This is thought to be due to the geographical location of different environments affecting semen quality. Tadesse et al. (2022) stated that the quality of spermatozoa DNA in cattle breeds is influenced by geographical location, which is related to environmental factors. Furthermore, environmental factors greatly affect the continuity of bovine spermatogenesis. This spermatogenesis is related to the temperature of the epididymis.

The data showed that the DNA quality in these three local cattle was good because the DNA damage was below 3% (Indriastuti et al. 2020). This value was lower than that reported in a previous study on local Pasundan cattle (3.56%) (Santoso et al. 2021). A study on DNA damage in *B. taurus* cattle in Indonesia using eosin showed a DNA damage of <10% (Prabowo et al. 2022). Using the Sperm–Bos–Halomax® report, the sperm DNA damage in Friesian-Holstein cattle was 17.89% (Enciso et al. 2011), whereas the sperm DNA damage in Brahman cattle was 4.17+2.69% (Priyanto 2015). Based on the research reported by these experts, the sperm DNA quality in the frozen semen of the local cattle in this study was good; however, it is still necessary to determine SDF as the main standard in frozen semen.

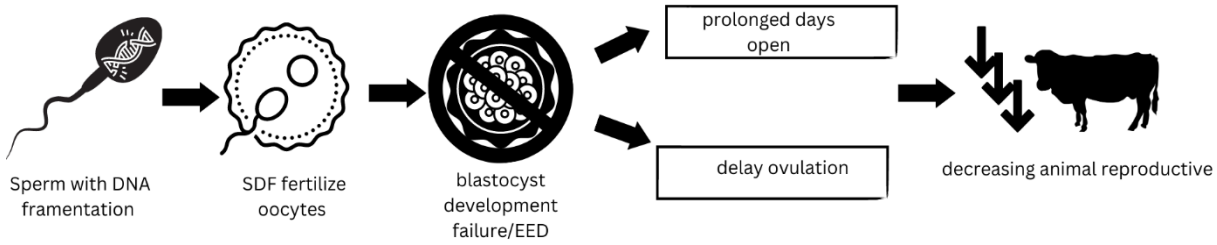

Figure 2. Illustration SDF declined cattle reproduction

In humans, SDF has already become the main requirement for sperm quality in AI or IVF procedures. The potential SDF refers to breaks in the genetic material (DNA) in the sperm that could still be expected to occur (Singh et al. 2020). It should also be adopted for ART in farm animals to increase the productive status of local cattle because it could enhance the potential of abortive apoptosis (conditions in which a damaged sperm cell evades predetermined cell death and remains in the ejaculate). Nevertheless, damaged spermatozoa with SDF retain their overall mating capability but cannot sustain gestation, leading to premature fetal death (Figure 2) (Kumaresan et al. 2017).

The chromatin quality in the nucleus largely determines the status of DNA tightly bound to protamine, which protects nuclear DNA (Priyanto et al. 2019). Therefore, changes in chromatin could change the DNA status (Sakkas and Alvarez 2010). This examination was based on the conditions of chromatin condensation, as indicated by the intensity of the resulting colors. This change determines the quality of the chromatin condensation process, which can be detected using Sperm–Bos–Halomax® (Figure 1).

DNA chromatin does not appear if condensation is more compact or intact. In contrast, colors appear in the chromatin in less compact or incomplete condensation. Incompact condensation is positively associated with chromatin abnormalities and sperm DNA integrity. This condition is triggered by various factors: exposure to free radicals during spermatogenesis, apoptosis, lack of proteins (especially arginine and cystine), infection, stress, exposure to toxic chemicals, testicular hyperthermia, and hormones (Fernandez-Novo et al. 2021). Research on DNA damage testing is very important, considering that one of the factors of embryo miscarriage in livestock occurs due to DNA damage factors. Farmers lose because the estrus cycle becomes longer due to the embryo miscarriage factor. Through DNA quality testing with simple methods, it is hoped that embryo cases in cattle will be reduced in the future so that the population can increase rapidly (Priyanto et al. 2019).

This research concludes that Aceh cattle had the highest post average compared to Bali and Madura cattle at 63.33±2.43%. The abnormality of Bali cattle had the lowest average compared to Aceh and Madura cattle at 8.83±1.04%. The evaluation of sperm DNA damage in local cattle using the Sperm-Bos-Halomax® method showed that the highest average DNA damage was 1.72±0.62% in Aceh cattle, and the lowest was 1.67±0.66% in Madura cattle. The sperm DNA damage in Bali cattle was 1.67±0.70%. The study's results stated that the livestock type affected the spermatozoa DNA quality ($P<0.05$). This is thought to be due to the geographical location of different environments affecting semen quality. Based on the research findings reported by these experts, the sperm DNA quality in the frozen semen of the local cattle in this study was good, and it is important to determine SDF as the main standard in frozen semen.

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