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Research Article
Detection of DNA Damage in Frozen Bovine Semen Using Eosin Staining

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Abstract

Background and Objective: Early detection of DNA defects in spermatozoa is vital because genetic abnormalities are associated with infertility and miscarriage. Eosin is an acidic stain with negatively charged chromatolytic components also found in the cytoplasm of spermatozoa. This study aimed to observe the effectiveness of eosin staining in detecting spermatozoa DNA deterioration. **Materials and Methods:** A total of four-hundred frozen beef straws from Simmental, Limousin and Brahman cattle and Ongole crossbreeds, were obtained from the Singosari National Artificial Insemination Center, Malang, Indonesia. Hydrogen peroxide was used to degrade frozen sperm. In addition, the sperm was subjected to three treatments: Agarose trapping, membrane lysis and eosin staining. **Results:** Damaged bull spermatozoa stained with eosin were bright red in color around the head and mid piece, whereas the head of undamaged spermatozoa were darker red. The average value of damaged DNA was 89.08±3.27% as assessed with eosin staining. **Conclusion:** Eosin staining effectively detects DNA damage in bull spermatozoa, but extended effectiveness tests are considered necessary.

Key words: Bull, damage, DNA, eosin, spermatozoa

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Artificial insemination (AI) is a breeding technique that has significantly impacted livestock productivity[1]. The success of AI is influenced by spermatozoa quality which is adversely affected by DNA damage[2]. Furthermore, spermatozoa DNA damage is exacerbated by male infertility and high rates of damage can have detrimental effects on ovum fertilization[3]. Accurate identification of spermatozoa DNA damage is therefore essential for the effectiveness of AI. AI technology used for breeding livestock can result in failed embryo development due to the transmission of damaged genetic material when using spermatozoa with high DNA damage values[4].

The DNA damage in spermatozoa can be identified by a variety of methods, including the terminal uridine nick-end labeling assay, sperm chromatin structure assay and comet assay[5]. However, most of these methods require complex or expensive instrumentation, such as flow cytometry and fluorescent microscopy and they are difficult to perform in developing countries such as Indonesia. The sperm nucleus with damaged chromatin contains histones that become rich in lysine residues and react positively to the dye. In contrast, sperm nuclei with intact chromatin contain more protamine, are rich in cysteine residues and react negatively to the dye[6]. The primary function of eosin staining in sperm is to distinguish mature and immature chromatin-containing sperm nuclei[7]. The objectives of this study were to determine the effectiveness of eosin staining for detecting DNA damage in bovine spermatozoa and to simultaneously determine the level of DNA damage using the eosin staining method.

MATERIALS AND METHODS

Study area: This study was conducted over a period of 5 months from July to November, 2021. This study used frozen bull semen (400 straws) from male Simmental, Limousin and Brahman cattle and Peranakan Ongole (PO) crossbreeds acquired from the Singosari National Artificial Insemination Center in Malang, Indonesia. All semen was produced on the same day and stored in a container containing liquid nitrogen at a temperature of -196°C, in accordance with Indonesian national standards. No animal ethics approval was required for this research since frozen semen were purchased from a commercial source. The study was conducted at the Animal Physiology and Reproduction Laboratory, in the Department of Animal Breeding and Reproduction, Faculty of Animal Science, at Gadjah Mada University, Yogyakarta, Indonesia.

All chemicals and reagents used in this study were obtained from Millipore Sigma (St. Louis, MO).

Data analysis: Spermatozoa DNA damage data were analyzed descriptively using images of eosin-stained spermatozoa. Data on the effectiveness of detecting spermatozoa DNA damage are presented as Mean±Standard error.

RESULTS

The study showed that eosin staining could detect chromatin damage in bull spermatozoa. Eosin staining revealed a bright red color around the head and mid piece of damaged spermatozoa, whereas, the heads of the undamaged spermatozoa were darker in color in Fig. 1a and b. The average spermatozoa DNA damage indicated by eosin staining was 88.38±3.06% from frozen semen damaged by H_2O_2 in Table 1.

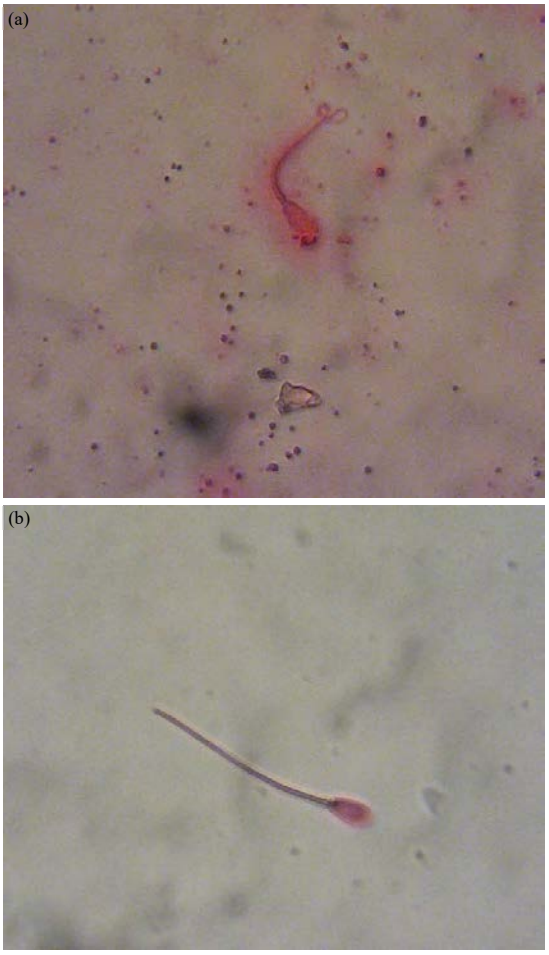

Fig. 1(a-b): Spermatozoa damaging, (a) DNA damage and (b) Without DNA damage

Table 1: Average number of bull spermatozoa that have been damaged

Bulls commodities	Number of frozen sperm whose DNA was damaged	Number of DNA damage identified by the eosin stain
Aceh	100	87.54±4.36
Madura	100	88.28±2.21
Ongole	100	88.50±1.83
Bali	100	89.20±3.84
Average	400	88.38±3.06

DISCUSSION

Eosin is used as a marker for damaged cells, only penetrating cells with damaged membranes and not those whose membranes are intact[8]. In this study eosin staining was used to determine bovine spermatozoa DNA damage (Table 1). Since eosin is acidic, it stimulates the release of acidophilic components in tissues such as in the mitochondria, secretory granules and collagen, therefore resulting in colored staining of these tissues[9]. The principle of staining is the affinity between the tissue and the reagent, either directly (the color material and the tissue bind directly) or indirectly (the color material and the tissue cannot bind directly unless given an intermediate material known as mordant)[10,11].

Spermatozoa DNA damage was defined by eosin color absorption. The resulting differences in color between damaged and undamaged spermatozoa (Fig. 1a, b) are thought to be caused by chromatin damage in the DNA. The eosin stain was developed based on the permeability of the dead sperm cell membrane for the dye, which is influenced by the nucleus quality[12]. According to Kondracki *et al.*[13], viable sperm would not absorb the stain because they have an undamaged nuclear envelope, whereas spermatozoa with damaged membranes would absorb the stain.

The chromatin quality in the cell nucleus significantly influences the status of DNA, which is covalently compacted to protamine that acts as a nuclear DNA protector[14]. Alterations in chromatin also result in changes in DNA status[15]. This study focused on chromatin condensation conditions manifested as distinctions in the intensity of the resulting color after eosin staining[16]. The brighter the color, the more compact or intact the condensation is and the darker the red color, the less compact or incomplete it is[17]. Uncompact condensation refers to DNA components that are not strongly attached and not densely packed as a result of imperfect transcription and translation processes[12]. It is associated with chromatin abnormalities and affects spermatozoa DNA integrity[18]. Several factors, including free radical exposure during the spermatogenesis process, apoptosis, a lack of arginine and cysteine, infection, stress, toxic chemical exposure, testicular hyperthermia and hormonal changes, could cause chromatin abnormalities[19].

Table 1 shows that using eosin as an indicator of sperm DNA damage was 88.38% effective, whereas 11.62% could not be detected, owing to the effectiveness of H_2O_2 in damaging the quality of spermatozoa by more than 90%. The H_2O_2 damages the DNA of spermatozoa through oxidation of various compounds in cells. It is destructive because it generates hydroxyl radicals and hypochlorite ions through reactions catalyzed by enzymes found in cell membranes[5]. The DNA chromatin becomes unstable resulting in weak spermatozoa DNA condensation and eventually causing damage[20]. Exposure to exogenous H_2O_2 adversely affects all sperm motility parameters. The H_2O_2 exerts a direct cytotoxic effect on spermatozoa.

The inactivation of intracellular scavenger systems after H_2O_2 treatment may be the cause of the decrease in cell viability. Exposure to high levels of H_2O_2 efficiently breaks duplex DNA and macerates the chromosome. This multifaceted toxicity potentiation by H_2O_2 results in robust and efficient damage[21]. Furthermore, it is thought that the non-staining of the damaged DNA was because of the affinity of eosin for some sperm cell nuclei not being able to bind directly optimally. Ransy *et al.*[22] discovered that, despite becoming increasingly static, the spermatozoa were alive and showed no signs of major membrane damage. Thus, the mechanism by which H_2O_2 affects sperm viability remains unknown.

Among the numerous studies on male infertility, spermatozoa DNA damage has attracted attention[10]. Defects in chromatin packaging during spermatogenesis can cause DNA damage in spermatozoa[23], apoptosis during spermatogenesis or genital tract sperm transport[8], or oxidative stress[24]. The protein concentration of spermatozoa DNA is negatively correlated with the level of DNA damage in spermatozoa[25]. Protein (protamine) deficiency diminishes spermatozoa viability, motility, morphology and tends to increase spermatozoa chromatin damage[6], decrease viability and increase DNA damage[26]. The DNA chromatin damage also shows a low success rate of in vitro fertilization[27]. The existence of protein deficiencies and irregularities during embryogenesis interrupts the development of the embryo and affects its survival[28].

CONCLUSION

The frozen semen storage process can increase spermatozoa DNA damage. To ensure the success of AI, it is necessary to test the quality of spermatozoa DNA. Eosin staining could be used to identify DNA damage in bovine spermatozoa using a light microscope; however, further research is necessary to confirm the efficacy of eosin staining in molecular research.

SIGNIFICANCE STATEMENT

This study found that eosin could be used as a detection material for DNA damage of bovine spermatozoa. This finding will help researchers and related parties such as AI centers and livestock nurseries to select superior males. Therefore, a new theory regarding the role of eosin in the detection of bovine spermatozoa DNA damage has been formed.

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