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The Concentration of Hydrogen Peroxide, Percentage of Motility, Viability, Plasma Membrane Integrity, and Abnormality of Frozen Semen of Horned and Polled Bali Bulls

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Abstract

The aim of the study was to determine the frozen semen quality of horned and hornless (polled) Bali bull including hydrogen peroxide (H_2O_2) concentration, motility, viability, plasma membrane integrity, and abnormalities. This study used frozen semen from 2 horned and 2 polled Bali bulls aged 5 to 7 years. The concentration of H_2O_2 was measured using 2-7-Dichlorodihydrofluorescein Diacetate (DCHF-DA) dye, motility was assessed subjectively under a microscope, viability was assessed by eosin-negrosin staining, plasma membrane integrity was calculated using the Hypoosmotic Swelling Test (HOS-Test), and abnormalities were evaluated with Sperm Stein Ready to USE stain.

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The results showed that there was no significant difference (P>0.05) between horned and polled based on the H_2O_2 concentration (2.37 ± 0.64 vs 2.65 ± 0.94), motility was (47.25 ± 5.04 vs 44.25 ± 8.12), plasma membrane integrity (50.62 ± 6.27 vs 52.75 ± 5.76), and abnormalities (15.90 ± 5.70 vs. 19.18 ± 6.39) but there was a significant difference (P<0.05) in the viability value (53.71 ± 8.56 vs. 64.09 ± 9.48). It can be concluded that the quality of frozen semen of horned and hornless (polled) Bali cattle based on H_2O_2 concentration, motility, plasma membrane integrity and abnormalities had the same conditions. The viability of spermatozoa from frozen semen of polled was better than that of horned Bali cattle.

Keywords: Horned and Polled Bali Bulls; Semen Quality.

1. Introduction

Bali cattle are beef cattle that are in great demand by breeders and the people because of their adapt ability to a marginal environment that some other breeds of cattle do not have [1]. The ability to adapt to the environment is an advantage of Bali cattle, so that its cultivation continues to be developed through crossbreeding to get a type of Bali cattle whose horns do not grow normally (polled) [2]. Bali cattle whose horns do not grow naturally (polled) were first discovered in Sidenreng-Rappang (Sidrap) in 1980 as a result of crossing Bali cattle with Brahman Cross [2]. This type of polled Bali cattle is considered to have advantages in the process of rearing. Efforts can be made to develop polled Bali cattle, one of which is reproductive biotechnology in the form of artificial insemination (IB).

The success of AI cannot be separated from the quality of frozen semen used, the low quality of frozen semen is generally due to damage to spermatozoa [3]. Spermatozoa that pass through the freezing and thawing processes cause the formation of ice crystals and osmotic pressure in the cells, thereby damaging the plasma membrane, decreasing motility, and triggering an increase in the production of Reactive Oxygen Species (ROS) [4]. The freezing process will cause dry conditions below the freezing point, so the solvent (water) will form ice crystals. The ice crystals will mechanically damage the spermatozoa and when the electrolyte concentration is excessive the lipoprotein envelope will dissolve, causing changes in cell membrane permeability and cell death. The thawing process can cause the process of releasing cryoprotectants to be inhibited, so that the spermatozoa are poisoned and cause a decrease in the ability to move spermatozoa [5]. Based on the explanation above, it is necessary to test the quality of spermatozoa from frozen semen of horned and polled Bali cattle, based on concentration of H_2O_2, motility, viability, plasma membrane integrity, and abnormalities.

2. Materials and Methods

2.1. Materials

The research was conducted at the In Vitro Embryo Production Laboratory, Institute for Research and Community Service, Hasanuddin University, Makassar. The samples used were frozen semen from 2 horned and 2 polled Bali cattle aged 5-7 years.

2.2. Methods

2.2.1. Thawing

Thawing is an attempt to thaw the frozen semen by dipping or soaking the straws in a water bath at 37°C for 15 seconds [6].

2.2.2. Concentration of Hydrogen Peroxide (H_2O_2)

Measurement of H_2O_2 concentration of frozen semen using 2', 7'-dichlorodihydrofluorescein diacetate (DCHF-DA, Sigma Co, USA) [7]. The staining procedure of 2', 7'-dichlorodihydrofluorescein diacetate (DCHF-DA, Sigma Co, USA) followed the procedure of Bateni and his colleagues [8], which involved making stock of 10 µM DCHF-DA stored at -70°C. Semen sample as much 10 µl was added with 5 µl of DCHF-DA, incubated for 30 minutes at 37°C, added PBS 300 µl then centrifuged twice. The sample was dripped on a glass object covered with a cover glass. Fluorescence emission was recorded with a digital camera (Zeiss AxioCam HRc, Germany) on fluorescence microscope (Zeiss Axio Imager A2) using 480 nm excitation and 510 nm emission. Fluorescent images were converted to TIFF files using Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA), then analyzed with ImageJ 1.47 software (Sun Microsystems, Inc., California, USA). The fluorescent image is measured by counting the number of pixels after the color inversion. The fluorescence intensity represents the intracellular concentration of H_2O_2.

2.2.3. Motility of Sperm

Motility is assessed subjectively by looking at the number of spermatozoa that move straight ahead (progressive), the standard of motility assessment is in the range of 0 - 100% [9].

2.2.4. Viability of Sperm

Viability was assessed by staining eosin-negrosin added to the semen sample and observed under a microscope at 400× magnification [10].

2.2.5. Plasma Membrane Integrity

Evaluation of the plasma membrane integrity of spermatozoa using the Hypoosmotic Swelling Test (HOS-Test) with a composition of 0.9/g fructose and 0.49/g sodium citrate dissolved in aquabides, 200 µl hypoosmotic solution incubated at 37°C for 35 minutes, then added 20 µl of semen which has been thawed, homogenized and allowed to stand again for 30 minutes [11]. Observations were made under a phase contrast microscope with a magnification of 400×, then 10 µl of spermatozoa that had been mixed with HOS-Test media dripped on a glass object and then covered with a cover glass, 200 spermatozoa cells were counted under a microscope with 400× magnification.

2.2.6. Abnormality of Sperm

Sperm abnormalities were evaluated with Sperm Stein Ready to USE dye, by dripping 10 µl of semen on a glass

object, making smear preparations, then drying. The preparations were soaked for 5 seconds in reagent 1 (methanol) followed by reagent 2 (Xanthene buffered solution), followed by reagent 3 (Thiazine buffered solution) and finally in distilled water, waiting for the preparations to dry and then observed under a microscope with 400x magnification in 5 fields of view or at least 200 cells, were evaluated with the help of oil immersion oil [12].

2.3. Data Analysis

The collected data was analyzed using the independent sample t-test.

3. Results and Discussions

Assessment of frozen semen quality is an attempt to determine the feasibility of stored frozen semen. This is because the freezing process will cause damage to the membrane structure and reduce motility and viability up to 25-75%, so this tester will determine whether the stored sample is still in a safe condition for use in the future [13]. The semen quality evaluated included hydrogen peroxide (H_2O_2) concentration, motility, viability, plasma membrane integrity and spermatozoa abnormalities from frozen semen of horned and polled Bali cattle. The results of quality testing carried out on frozen semen of horned and polled Bali cattle are presented in Table 1.

Table 1: The quality of frozen semen of horned and polled Bali cattle

Groups	H_2O_2 (FIU)	Motility (%)	Viability (%)	PMI (%)	ABN (%)
Horned	2.37 ± 0.64	47.25 ± 5.04	53.71 ± 8.56[a]	50.62 ±6.27	15.90 ± 5.70
Polled	2.65 ± 0.94	44.25 ± 8.12	64.09 ± 9.48[b]	52.75 ± 5.76	19.18 ± 6.39

[ab]Different superscripts in the same column showed significant differences (P<0.05). FIU (Fluorescence Intensity Units); PMI (Plasma Membrane Integrity); ABN (Abnormality).

3.1. Concentration of Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide is part of reactive oxygen species (ROS), most of which are the result of cell metabolism and external exposure. Excessive hydrogen peroxide formation will cause cells to experience oxidative stress, which will trigger cell tissue damage [14]. Measurement of the H_2O_2 concentration of spermatozoa from frozen semen of horned and polled Bali cattle used 2', 7'-dichlorodihydrofluorescein dye. The result of the staining will be a green glow on the surface of the spermatozoa, which spermatozoa with a high concentration of H_2O_2 will glow a bright green color, as shown in Figure 1. This is in accordance with the opinion of Kiani-Esfahani and his colleagues [7] and Prihantoko and his colleagues [15] that the intracellular flowcytometry method using 2', 7'-Dichlorodihydrofluorescein Diacetate (DCHF-DA) is considered capable of detecting hydrogen peroxide content and ROS activity in spermatozoa cells, because of the nature of DCHF-DA will enter passively into cells by the action of cellular esterases. The presence of oxidative substances (hydrogen peroxide) DCHF will be oxidized to dichlorofluorescein (DCF) and will glow green fluorescence (color) in spermatozoa samples.

The measurement of H_2O_2 concentration in frozen semen of horned and polled Bali cattle was not significantly different ($P>0.05$) as in Table 1. The results of this study showed that the semen of horned and polled Bali bull had the same ability to prevent the formation of free radicals during the frozen semen manufacturing process, although it exposed to higher oxygen concentrations after collection until the freezing process. Gustina and his colleagues [16] explained that the oxygen concentration in in vitro environmental conditions is very different from in vivo conditions. It was further explained that in in vitro conditions the oxygen concentration could reach 20% while in vivo only ranged from 3% to 9%. Measurement of H_2O_2 concentration in this study was lower than the previous study reported by Gürler and his colleagues [17] on frozen semen of Simental cattle (23.02-80.09), Gallo and his colleagues [18] in frozen semen of FH cattle (7.0-27.9) and human (13.6-96.5), and Mislei and his colleagues [19] on frozen horse semen (185.1-379.2). According to Anwar and his colleagues [20] that the energy required of spermatozoa during the incubation process increased along with the intensity of cell metabolism, which eventually led to an increase in oxygen consumption of spermatozoa. The increase in the intensity of lipid peroxidation in cells is accompanied by an increase in oxygen consumption in cells and the formation of H_2O_2 radicals [21].

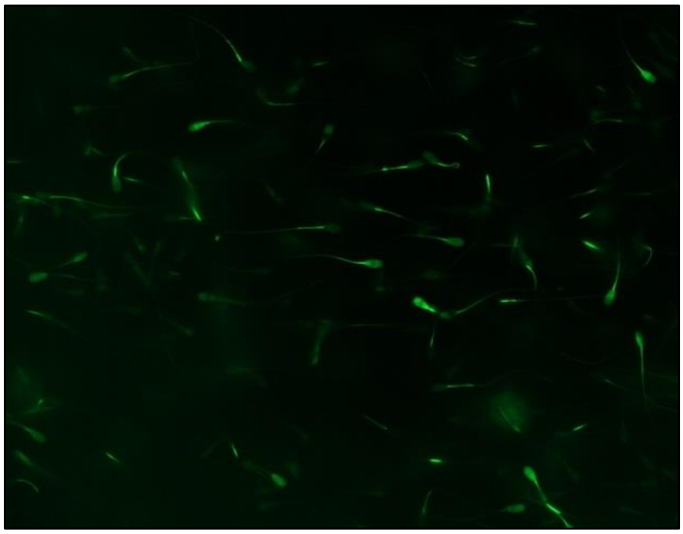

Figure 1: Photomicrograph of spermatozoa with 2',7'-dichlorodihydrofluorescein diacetate (400× magnification).

Spermatozoa handling processes such as washing, removal of seminal plasma semen, and centrifugation can damage and eliminate the antioxidants in spermatozoa, so that ROS can increase. Spermatozoa can utilize ROS when they are at normal level as activation during the fertilization process, but when ROS is in an excessive condition, it can make spermatozoa fail to fertilize. Specifically, damage to spermatozoa caused by ROS, can affect the integrity of the spermatozoa genome, make lipid peroxidation, and lose the ability to decrease fluidity and motility [22].

3.2. Motility of Sperm

Spermatozoa motility was assessed from the spermatozoa that were able to move progressively. The percentage

of frozen semen motility of horned and polled Bali cattle was not significantly different (P>0.05) (Table 1). However, in this study spermatozoa from frozen semen of polled Bali cattle tends to have lower motility values than horned. Nevertheless, frozen semen of polled and horned is still suitable for use in the AI program. It is based on the National Standardization Agency [23] that frozen semen that is suitable for use must have a spermatozoa motility of at least 40%. The results of this research are lower than previous studies reported by Yendraliza and his colleagues [24] on frozen semen of Bali cattle with 60.66-66.66 value.

The semen freezing process can reduce the motility ability of spermatozoa. This is because the fresh semen obtained during storage will experience significant temperature changes, both during the freezing and thawing processes. Nofa and his colleagues [25] explained semen freezing will make spermatozoa experience excessive osmotic stress and cold shock, resulting in decreased mobility. Increased metabolism in spermatozoa, along with increased lactic acid production, so that the pH decreases which causes low mobility of spermatozoa [26]. Decreased motility after thawing can be caused by spermatozoa experiencing cryoprotectant poisoning. This occurs because the process of releasing cryoprotectants in cells is inhibited, causing a decrease in the ability to move spermatozoa [5]. The motility of spermatozoa is highly dependent on the energy possessed by the spermatozoa because when they are fresh, the spermatozoa are mixed with seminal plasma which contains organic compounds such as fructose, glucose, and sucrose [27].

3.3. Viability of Sperm

Viability is an assessment of the motile (live) and immotile (dead) spermatozoa cells. The viability test in this study used eosin-negrosin dye, in which dead spermatozoa will absorb the eosin color, while the live spermatozoa will colorless [28]. The picture of the viability of the spermatozoa tested in this study is shown in Figure 2.

The spermatozoa viability of frozen semen of polled Bali cattle was significantly higher (P<0.05) than horned (Table 1). This indicates that the frozen semen of polled Bali bull is more resistant to temperature changes during the semen processing and the thawing process than horned. Pubiandara and his colleagues [29] explained that drastic changes in temperature can result in cold shock in spermatozoa. Cold shock can result in changes in the phospholipids that make up the plasma membrane of spermatozoa. Changes that occur in plasma membrane phospholipids can cause damage to the membrane. However, the percentage of spermatozoa viability of horned is still above 50% which indicates that it is still suitable for use in the AI process. Pardede and his colleagues [13] described frozen semen suitable for use in the Artificial Insemination (AI) reproductive biotechnology, at least 50% of spermatozoa were alive or motile. Ardhani and his colleagues [26] also studied frozen semen of Bali cattle which was stored for 10 years and obtained a viability value of 55.33±2.60%.

The quality of spermatozoa will decrease due to the freezing process, but the quality of frozen semen is also determined by the quality of the spermatozoa when they are still fresh [25]. Ardhani and his colleagues [26] reported that the freezing process of spermatozoa will reduce the viability of spermatozoa. The freezing process will cause dry conditions below the freezing point, so that the solvent (water) will form ice crystals, the ice crystals will mechanically damage the spermatozoa and when the electrolyte concentration is excessive so the

lipoprotein sheath will dissolve, causing changes in cell membrane permeability and cell death. Cojkic and his colleagues [30] argued that the freezing process in spermatozoa cells would cause cellular dehydration, resulting in a decrease in motility and viability values up to 25-27%.

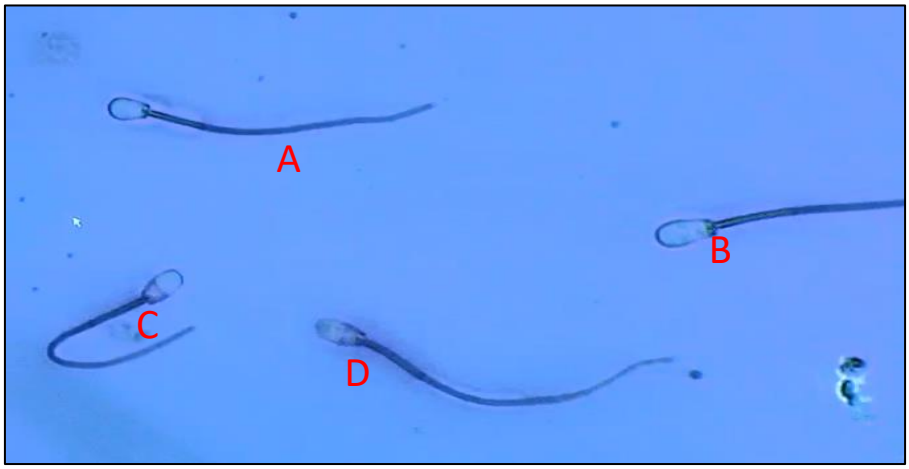

Figure 2: Observation of spermatozoa viability with eosin-negrosin. Motile spermatozoa (A and B), immotile spermatozoa (C and D) with 400× magnification.

3.4. Plasma Membrane Integrity

The plasma membrane integrity of spermatozoa measured by the Hypoosmotic Swelling Test (HOS-Test) solution. In this measurement, the straight sperm tail indicating the condition of the spermatozoa membrane is damaged, whereas in spermatozoa with intact membranes, the tail will be coiled or inflated as shown in Figure 3. This is due to the solution used in the test, which creates osmotic pressure around the spermatozoa cells so that when hypoosmotic fluid enters intact cells, it will make it difficult for water to escape, whereas in cells whose membranes are not intact, spermatozoa hence the hypoosmotic fluid easily enters and leaves the cell membrane [30].

The percentage of plasma membrane integrity (PMI) in this study was not significantly different ($P>0.05$). Horned Bali cattle tend to have a higher PMI percentage than polled (Table 1). This indicated that the plasma membrane of polled Bali cattle spermatozoa has higher resistance to temperature changes during processing and thawing compared to horned. Spermatozoa that pass through the freezing process and thawing will cause drastic temperature changes, lipid peroxidation, and membrane damage. Arvioges and his colleagues [11] and Uttama and his colleagues [31] explained that during the thawing process, free radicals of oxygen metabolites are formed which are toxic to spermatozoa and cause limited oxygen supply, so that oxygen utilization by spermatozoa increases, which can lead to increased production of free radicals, lipid peroxidation and damage to spermatozoa membranes. The plasma membrane of spermatozoa plays a very important role because it affects the metabolism of spermatozoa. Cardullo and Florman [32] explained that the plasma membrane of spermatozoa plays a role in protecting all cell organelles. The percentage of PMI in this study was not much different from the test described by Arvioges and his colleagues [11] in cattle that the percentage of PMI ranged 17.83-59.50. Furthermore, Pardede and his colleagues [13] explained that intact plasma membranes play an important role in

the process of capacitation, acrosome reactions and oocyte fertilization.

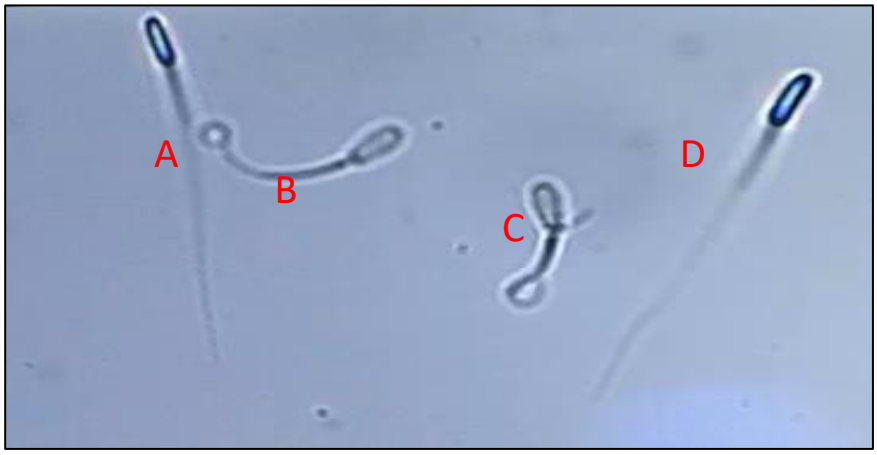

Figure 3: Observation of the plasma membrane integrity value of spermatozoa using the HOS-Test method. Spermatozoa A and D (Damage Plasma Membrane), Spermatozoa B and C (Intact Plasma Membrane) with 400× magnification.

3.5. Abnormality of Sperm

Spermatozoa abnormalities consist of head, neck and tail. The percentage of abnormality of spermatozoa in horned and polled Bali cattle did not differ (Table 1.) This indicates that the spermatozoa of horned and polled Bali cattle have similarity in endurance during the freezing process. The percentage of abnormalities in this study was still in normal conditions. This is in accordance with the report of Kumar and his colleagues [33] that maximum abnormality of spermatozoa in cattle is 20%. Indriastuti and his colleagues [34] explained that spermatozoa abnormalities can occur due to disruption during the process of spermiogenesis or during the maturation process in the epididymis.

Abnormalities of spermatozoa were found in this study include circular tail and thick neck, simple bent tail, double tail, and head damage. Utami and Tophianong [35] found spermatozoa abnormalities of FH cattle in the form of microcephalic (bent tail, proximal droplet and simple bent tail). The abnormality of the tail occurs due to the modification of the flagellum when the spermatozoa are in the scrotum to the epididymis. This is also influenced by heat on the scrotum [36]. Measurement of spermatozoa abnormalities in Aceh cattle has also been carried out by Rahmiati and his colleagues [37] that 13, 27, 9, 18 and 8.58% results of different ejaculation frequencies, so that the frequency of repeated ejaculation at one time of storage could reduce the morphological quality of spermatozoa. Spermatozoa produced from the reproduction of male mammals such as cattle, but there will be differences in the morphology of spermatozoa in both the head and tail due to environmental influences [38].

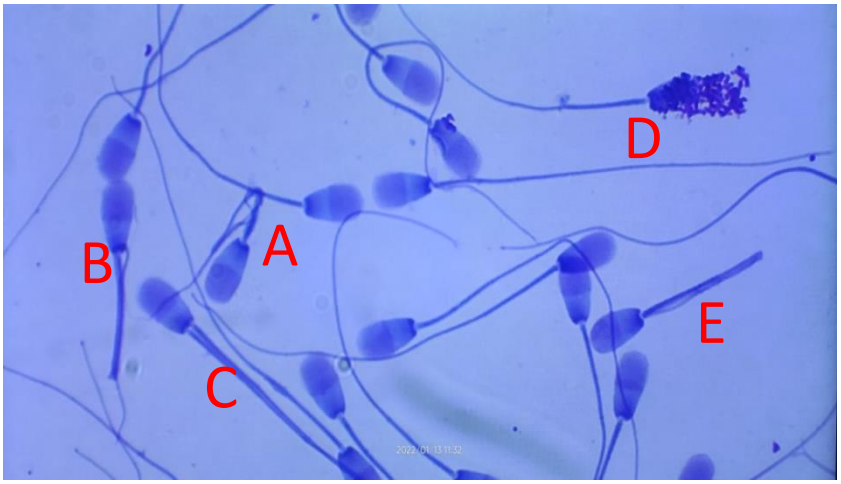

Figure 4: Spermatozoa with abnormal morphology are shown in letter A, B, C, D, E (100x magnification).

4. Conclusion

H_2O_2 concentration, motility, plasma membrane integrity, and abnormalities in frozen semen of horned and polled Bali cattle had the same conditions. The frozen semen viability of polled Bali cattle is better than that of horned.

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