The Acrosome Integrity Examination of Post-thawed Spermatozoa of Several Ongole Grade Bull in Indonesia Using Giemsa Staining Method

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Abstract. Cement cryopreservation is a preserving technique of spermatozoa cells that the spermatozoa cells can be used in a long period of time. Frozen semen is produced by the National Artificial Insemination Center (AIC) and the Regional Artificial Insemination Center (RAIC) in Indonesia. The indicators in predicting spermatozoa fertilizing ability include individual motility and movement scores, but the indicators have not been able to accurately predict the spermatozoa fertilizing ability. Acrosome integrity of spermatozoa cells is an important indicator of the success of the fertilization. The study aimed at evaluating the quality of the acrosome integrity of Ongole Grade Bull post-thawed spermatozoa from several Artificial Insemination Centers (AIC) in Indonesia using Giemsa staining method. It used 60 straw samples of 6 PO cattle aged 6 to 8 years with healthy and normal reproductive organs obtained from 3 different Artificial Insemination Centers (AICs) in Indonesia. The spermatozoa acrosome integrity was evaluated using Trypan Blue-Giemsa (TBG) and Giemsa stainings combined with fixation using methanol. The results showed that the motility (43.60±1.65%) and the viability of the spermatozoa (55.75±1.83%) of Ongole grade bull in the AIC A was higher than those in the other AIC (P<0.05). The results of the evaluation of the acrosome integrity of the Ongole grade bull from the AIC A using the TBG staining showed that the acrosome integrity of the bull from the AIC A was higher than that of the bull from the other AIC (P<0.05) (77.00±1.52%). They also showed that the acrosome integrity of the Ongole grade bull from the AIC A evaluated using Giemsa staining was also higher than that of the bull from the other AIC (P<0.05) (76.84±1.57%). Spermatozoa acrosomes can be evaluated using TBG or Giemsa stainings. However, the TBG staining is more complex and it takes a long time for the staining to give expected results. Meanwhile, the Giemsa staining combined with fixation using methanol is a simple method and it does not take a long time for the staining to give satisfying results.

1. Introduction

Cryopreservation is a technique of preserving animal or plant cells, or other genetic materials (including semen) in a frozen state (-196 °C) by reducing metabolic activity without affecting the organelles found in the cells and hence maintaining the physiological, biological, and morphological functions [20]. Frozen semen is the product of spermatozoa cryopreservation and at present the frozen semen is produced by National Artificial Insemination Center (NAIC) and Regional Artificial Insemination Center (RAIC). It has to meet the quality standard established by National Standardization
Agency (NSA) as stipulated in Indonesian National Standard (INS) No. 4869.1: 2008 on frozen bull semen [34]. The indicators in testing the quality of the frozen semen according to the quality standards set in the INS are only motility and individual motion. The predicting ability of semen fertility of the laboratory tests is still limited, especially because of the complexity of sperm and fertilization process. Therefore, it is necessary to increase the accuracy of the prediction of bull fertility using other tests that the accuracy of the reproductive potential estimate of the bull is in turn improved. In fact, another equally important indicator that can be specifically used to determine the fertilizing ability of spermatozoa is acrosome integrity of spermatozoa.

Motility and individual motion scores serve as indicators in predicting the fertilizing ability of spermatozoa, but the indicators are still unable to precisely indicate the fertilizing ability of the spermatozoa. The motility and the individual motion of the spermatozoa only indicate that live spermatozoa are able to move normally and pass through female reproductive tract. [26] stated that it is necessary for spermatozoa to be acrosome-intact to achieve successful fertilization by activating the function of acrosome reaction at the right time, releasing enzymes and facilitating spermatozoa to penetrate zona pellucida. Sperm freezing process will result in the exposure of spermatozoa to low temperature in liquid nitrogen (-196 oC). The freezing process will cause cold shock, oxidative stress and formation of ice crystals.

Spermatozoa have three types of membranes, which are plasma membrane, mitochondrial membrane, and acrosomal membrane. The membranes contain polyunsaturated fatty acids and hence are very susceptible to oxidative stress, especially during freezing procedures [8]. Oxidative stress is one of the factors that increase cell damage due to reactive oxygen species (ROS). Excessive ROS production in sperms is dangerous due to its negative effects on functional sperm count [30]. The membrane of mammalian spermatozoa is susceptible to oxidation due to the presence of ROS because it is rich in unsaturated fatty acids. Such lipid peroxidation chain reactions take place continuously (autocatalytic) since each reaction produces a new reactive oxygen species (ROS) that leads to a new lipid peroxidation reaction and finally damages the entire plasma membrane of spermatozoa cells. The lipid peroxidation can change membrane functions and hence decrease the metabolism, the morphology, the motility and the fertility of sperms [28]. Acrosome integrity is one of the determinant factors of the success of fertilization. Only acrosome-intact spermatozoa are able to penetrate the zona pellucida and fuse with oocyte plasma membrane [7].

The acrosome integrity of spermatozoa can be observed using various staining methods such as Trypan Blue-Giemsa (TBG) and Coomassie Blue (CB). TBG staining has been used in examining the acrosome of the spermatozoa of various species such as bull and pig [21], while CB staining has been reported on the spermatozoa of rabbits [22] and human [12]. Both staining methods have high affinity for protein and are expected to be capable of staining the acrosome of spermatozoa. TBG is staining capable of binding to cell protein. Giemsa is known to have low molecular weight and able to pass through cell membrane that protects the acrosome of spermatozoa. A previous study [27] showed that TBG staining is considered to be simpler and easier than CB staining. In addition to the two abovementioned staining methods, another study [2] has been conducted to assess the acrosomal integrity of spermatozoa using giemsa staining, which was a simpler and easier method, by combining methanol fixation and giemsa staining. Given that acrosomal integrity is very crucial in fertilization processes, the study aimed at evaluating the quality and acrosomal integrity of frozen spermatozoa of Ongole grade bulls from several AICs in Indonesia and determining the most effective staining method in assessing the acrosomal integrity of frozen spermatozoa.

2. Materials and methods

2.1. Materials
The main materials in study were 60 straw samples of PO bulls of 6 healthy Indonesian Ongole grade bulls aged 6 to 8 years with normal reproductive organs obtained from three different Artificial Insemination Centers (AICs).
2.2. The thawing of frozen semen
The frozen semen was removed from the liquid nitrogen container using tweezers, put into a microtube, and thawed using a water bath at the temperature of 37°C for 15 seconds.

2.3. The motility of spermatozoa
Spermatozoa motility analysis was made to find out the number of spermatozoa moving forward. Motility test was carried out by dropping 20 μL semen with a micropipette on an object glass and then the object glass was covered using a cover glass. The percentage of sperm motility was assessed from 0 to 100% in an estimate from five viewpoints by comparing the number of spermatozoa moving forward. The sperm motility was evaluated using a microscope at 100x magnification equipped with a heating table (37°C).

2.4. The viability of spermatozoa
Sperm viability was tested using eosin-nigrosin staining method. The sperm viability (%) was observed on the basis of live spermatozoa and their intact membrane structure and hence it prevented stain from entering sperm membrane. Meanwhile, the membrane structure of dead spermatozoa did not function for any longer that stain could enter the sperm membrane. The procedure was started by dropping a drop of semen on an object glass. Two drops of eosin-nigrosin staining reagent were added and then stirred until the mixture was homogenous. Smear preparation was made using another object glass. Fixation was done on Bunsen and then the smear preparation was observed using a microscope at 400x magnification.

2.5. The assessment of the membrane integrity of spermatozoa using HOST method
The membrane integrity of spermatozoa was analyzed using hypo osmotic swelling test (HOST) [16] with a slight modification using a solution of the composition of 0.9 g fructose and 0.49 g Sodium citrate dissolved in distilled water to get the volume of 100 ml. Two hundred μl of the solution was added to 20 μl of semen and then the semen was incubated at 37°C for 45 minutes. Next, smear preparation was made on an object glass. Subsequently, the preparation was immersed in methanol solution for 10 minutes, rinsed in running water, and dried. Finally, at least 100 spermatozoa were observed under a microscope at 400x magnification. Membrane-intact spermatozoa were characterized by swollen tail, while sperms with damaged membrane were characterized by straight tails.

2.6. The assessment of the acrosomal integrity of spermatozoa using Trypan Blue-Giemsa (TBG) staining method
The acrosomal integrity of spermatozoa was analyzed using Trypan Blue-Giemsa (TBG) staining method [21]. The TBG staining method was applied by dripping semen and trypan blue solution dissolved in 0.81% NaCl simultaneously and then slowly homogenized. Smear preparation was made and dried in vertical position. The preparation was stained using neutral red solution of the composition of 86 mL HCl 1.0 N, 14 mL 37% formaldehyde, 0.2g neutral red). It was stained for 2-5 minutes by evenly applying the solution on the preparation surface and then dried. Next, the preparation was rinsed in running water and dried once again. The next staining step was to immerse the preparation in a staining jar containing 5% Giemsa solution and left at room temperature for 3 days. It was rinsed once again by immersing it in a container containing water for 2 minutes. Once it has dried, it was warmed up using a heating table (40 °C). It was covered using a cover glass. Two hundred cells were evaluated under a microscope at 400x magnification. Acrosome-intact spermatozoa were characterized by purple head, while those with damaged acrosome were characterized by pale lavender.

2.7. The assessment of the acrosomal integrity of spermatozoa using giemsa staining method
The acrosomal integrity of spermatozoa was analyzed using Giemsa staining method [2]. The staining process was carried out by dripping semen on an object glass. Smear preparation was made and warmed using a warming plate at the temperature of 37 °C. The preparation was fixed using methanol for 10
minutes and then rinsed in running water. It was stained using Giemsa solution of the composition of 3 ml absolute Giemsa, 2 ml PBS, and 35 ml distilled water for 3 hours by immersing it in a staining jar. It was rinsed in running water and dried again. Two hundred cells were examined under a light microscope at 400x magnification. Acrosome-intact spermatozoa were characterized by purple head, while those with damaged acrosome were characterized by pale lavender head. Acrosomal status was established by counting the number of acrosome-intact spermatozoa divided by the total number of spermatozoa and multiplied by 100%.

2.8. Data analysis
Data were descriptively analyzed using Statistical Product and Service Solution (SPSS) version 24. The data included the motility, the viability, the plasma membrane integrity and the acrosome integrity of spermatozoa of the straw samples of Ongole grade bulls.

3. Results
The results of the study (Table 1) showed that the mean post-thawing motility of Ongole grade bulls was in the range of 39.85±1.32% to 43.60±1.65%. Post-thawing motility is used in the evaluation of cement after freezing to determine the quality and the quantity of frozen semen. Spermatozoa motility will decrease due to the freezing process because the thawing process of frozen spermatozoa will result in some spermatozoa with low viability and less progressive motility than those of fresh semen [1]. However, the frozen semen can be inseminated because it has met the quality requirements of frozen semen regulated in INS 4869.1: 2008, requiring that the motility of post-thawing spermatozoa should be at least 40%. The spermatozoa after freezing and thawing treatments will move more slowly than fresh semen. The difference in the speed is due to the application of diluents such as skim and egg yolk, which will result in the slower movement of the post-thawed semen.

Table 1 summarized the percentages of the spermatozoa motility of the Ongole grade bulls in three AICs. The spermatozoa motility of the Ongole grade bulls from the AIC A was higher than that of other AICs, and there was a significant difference in the spermatozoa motility between the AIC A and the other AICs (P<0.05).

The results showed that the mean viability of the spermatozoa of the Ongole grade bulls was in the range of 49.15±1.52% to 55.70±1.83% (Table 1). The spermatozoa viability was examined using eosin-nigrosin smear preparation able to color the head of dead spermatozoa red, while it will not give any color to the head of living spermatozoa. It was because the eosin could penetrate dead cell membrane and could not penetrate living cell membrane, while as background dye nigrosin would make the head of colorless sperm visible [4].

Table 1 summarized the percentages of the spermatozoa viability of the Ongole grade bulls in three AICs. The percentage of the spermatozoa motility of the Ongole crossbreed cattle from the AIC A and the AIC B was relatively higher than that of the spermatozoa motility of the cattle from AIC C. There was a significant difference in the spermatozoa motility of the cattle between the AIC C and the other AICs (the AIC A and the AIC B) (P <0.05).

Plasma membrane integrity is an absolute necessity for spermatozoa. Plasma membrane serves as first defense of cells against external environment that can damage the cells. The examination results showed that the mean spermatozoa membrane integrity was in the range of 50.90±1.32% to 70.22±1.55% (Table 1). The membrane integrity was examined using Hypo Osmotic Swelling Test (HOST) with citrate and fructose solution. An intact plasma membrane is characterized by a circular tail, while the spermatozoa with damaged plasma membrane are characterized by a straight tail due to increased permeability that enables hypotonic to enter the membrane so that the head appears bulging and the tail is straight.

Table 1 summarized the percentages of the spermatozoa viability of the Ongole grade bulls in three AICs. The percentage of the membrane integrity in the AIC A was higher than that in other AICs and there are significant differences in the membrane integrity between the AIC A and the other AICs (P<0.05). The percentage of the membrane integrity in the AIC A and the AIC B was relatively similar, while
there was not any significant difference in the membrane integrity between the AIC B and the AIC C (P>0.05).

The success of fertilization is determined by the presence/the status of acrosome, which is able to activate the function of the acrosome reaction and the fusion of oocyte. Table 2 summarized the percentages of the intact acrosome of the Ongole grade bulls from three Artificial Insemination Centers. Table 2 also summarized the percentages of the intact acrosome of the bulls in all AICs identified using TBG or Giemsa with the values between 62.65±0.50 to 77.00±1.52%. The TBG and Giemsa stainings showed relatively the same results, meaning that the percentage of the intact acrosome of the Ongole grade bulls from the AIC A was higher than that of the bulls from other AICs, while there was a significant difference in the percentage of the intact acrosome of the bulls between the AIC A and the other AICs (P<0.05).

4. Discussion

4.1. The spermatozoa quality of post-thawed frozen semen

After freezing and thawing, spermatozoa moves more slowly than fresh semen. One of the causal factors of such difference is the fact that the frozen semen with skim milk egg yolk extender moves more slowly. Additionally, freezing process also damages spermatozoa and it makes their movement slower. The decrease in the motility of the spermatozoa after freezing and thawing may be in the range of 24-64% [29]. The reason why the motility decreases is that the spermatozoa experience cold shocks and excessive osmotic stress. The decrease in the motility relates to mitochondrial activity [9]. Mitochondrial damage leads to disruption in the production of adenosine triphosphate (ATP). Mitochondria are the main sources of cell oxidative energy in the production of the ATP through electron transport system [33] and it adds the energy in the form of the ATP resulting from the metabolism process in
mitochondrial membrane that plays a role in microtubule activity, which enables spermatozoa to move freely.

The quality of frozen semen is not only affected by freezing processes, but also closely relates to the quality of fresh semen prior to freezing process. The effort to increase motility is made by feeding bulls with adequate quality forage and providing good management and maintenance of barns [32]. In fact, the quality of the semen of each bull is different even though the maintenance and feed management are the same [34]. The quality of fresh semen relates to the quality of frozen semen. Thus, the differences in the quality of the frozen semen obtained from the same or different breeds of bulls and from the same or different insemination centers may be influenced by the abovementioned factors.

Plasma membrane integrity is vital for spermatozoa because the plasma membrane integrity plays an important role in regulating all of the processes in cells [31]. The percentage of the sperm membrane integrity of the bulls from both insemination centers could be categorized as good. Phospholipids found in skim milk egg yolks play a role in protecting the plasma membrane from the effects of cold shock [23]. Phospholipids fuse through the sperm membrane, replace the damaged phospholipids of some membranes and hence reduce the effects of the damage due to temperature changes [17]. Skim milk contains lipoprotein and lecithin. Therefore, it can be used as semen extender to protect spermatozoa from the effects of cold shock.

4.2. The spermatozoa acrosome integrity of frozen semen

In all mammals, the capacitation and the subsequent acrosome reaction of spermatozoa represent essential steps for successful fertilization and formation of a zygote. The determination of the ability of spermatozoa to activate the acrosome reaction is supposed to be a useful parameter in evaluating infertility [19]. One of the key processes in mammalian fertilization is the acrosome reaction (AR) usually triggered in spermatozoa upon their binding to the zona pellucida of the egg. The AR involves fusion between the plasma membrane and the underlying outer acrosomal membrane, which results in the release of the acrosomal contents [10]. Acrosomes play a crucial role in the process of fertilization. Spermatozoa binding to the zona pellucida will stimulate acrosome reaction and cause the release and the activation of acrosome enzymes, allowing spermatozoa to penetrate the zona pellucida [24]. The difference in the obtained acrosome-intact values relates to the function of the membrane to protect spermatozoa acrosome. It is known that cholesterol is one of the components of spermatozoa membranes. Previous study [25] reported a significant decrease in the cholesterol in spermatozoa membrane after freezing. The cholesterol in the membrane functions as a capacitating agent. The amount and the distribution of the cholesterol in the spermatozoa membrane indicate capacitation steps [36]. The depletion of the cholesterol in the membrane is the first capacitation step characterized by the decrease in membrane stability [35].

The depletion of cholesterol in the membrane can increase membrane permeability to intracellular calcium and bicarbonate to activate adenylyl cyclase (AC), which produces cAMP and activates protein kinase A (PKA). Protein kinase opens ion channels in the membrane and modulates an increase in intracellular calcium [5]. At this step of capacitation, there is a change in enzyme activity and the motility of spermatozoa that are responsive to acrosome reaction [3]. Neild et al., [26] suggested that acrosome reaction is a process of releasing a penetrating enzyme that allows spermatozoa to penetrate zona pellucida and to fertilize oocytes. However, if the acrosome reaction takes place before spermatozoa reach fertilization site, the spermatozoa will lose their ability to fertilize oocytes. Spermatozoa must be acrosome-intact to have the ability to fertilize oocytes. Acrosome intactness can be tested using Giemsa and TBG stainings illustrated in Figure 1 and Figure 2.

The results of spermatozoa TBG staining showed that Giemsa staining has the ability to bind to membrane proteins. So, it can be assumed that the Giemsa can stain the membrane proteins. The damaged acrosome can be observed on the basis of the results of spermatozoa staining, whether they absorb color or not. In fact, several AICs produced the frozen semen using skim milk egg yolk extender so that the staining results were not good. The results would not be as good as those produced by the AIC that used Tris extender. The TBG staining was not quite effective in assessing frozen semen because
the extender components were also stained, which caused the difficulty in analyzing the results of the staining. Additionally, the analysis of the TBG staining results was more time consuming than that of simple Giemsa staining results.

Based on the results of the study, it can be concluded that the characteristics and the status of the spermatozoa acrosomes of the Ongole grade bulls from the three AICs are different. The examination of the acrosome integrity of the semen sample using the Tris extender gives clearer results. The analysis of the acrosomal status using the Giemsa staining is more efficient than that using the TBG staining.

**Figure 1.** The results of the acrosome staining with Trypan Blue-Giemsa (TBG) staining method (A) Intact acrosome or good acrosome integrity, (B) incomplete acrosome or poor acrosome integrity

**Figure 2.** The results of the acrosome staining with Giemsa staining method (A) Intact acrosome or good acrosome integrity, (B) incomplete acrosome or poor acrosome integrity

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