

## Evaluation of semen quality of buffalo frozen semen produced by Artificial Insemination Center

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### ABSTRAK

Penelitian ini bertujuan untuk mengevaluasi dan membandingkan kualitas spermatozoa *post thawing* dari semen beku kerbau produksi balai inseminasi buatan (BIB) terhadap nilai standar dan usulan nilai acuan (*proposed reference values*, PRV). Materi yang digunakan sebanyak 60 sampel *straw*, berasal dari tiga balai yang masing-masing 20 *straw*. Parameter yang diamati adalah motilitas, konsentrasi, longivitas, membran plasma utuh (MPU), tudung akrosom utuh (TAU) dan *recovery rate* (RR). Data yang diperoleh di uji Z kemudian disajikan dalam bentuk rata-rata  $\pm$  standar deviasi. Hasil penelitian dari ketiga balai inseminasi buatan menunjukkan nilai rata-rata motilitas  $45,00 \pm 3,07\%$ ; konsentrasi  $26,09 \pm 3,11 \times 10^6$  sel/0,25 mL; longivitas  $10,38 \pm 0,75\%$ /jam; MPU  $45,86 \pm 10,67\%$ , TAU  $73,99 \pm 5,28\%$  dan *recovery rate*  $64,38 \pm 5,16\%$ . Simpulan dari hasil penelitian ini adalah bahwa nilai rata-rata motilitas dan konsentrasi pada ketiga balai lebih tinggi dibandingkan dengan nilai Standar nasional Indonesia (SNI). Nilai rata-rata longivitas lebih rendah sedangkan nilai rata-rata MPU dan TAU lebih tinggi daripada standar nilai *Department of Animal Husbandry, Dairying and Fisheries* (DADF) dan nilai rata-rata *recovery rate* lebih tinggi dibandingkan usulan nilai acuan.

*Kata kunci: kualitas semen, spermatozoa, post thawing, kerbau*

### ABSTRACT

This study aimed to evaluate and compare the quality of post thawing spermatozoa of buffalo frozen semen produced by artificial insemination centers on standard values, and proposed reference values (PRV). Materials of the research were 60 samples of straws obtained from three Artificial Insemination Center, which are each 20 straws, respectively. Parameters observed were motility, concentration, longevity, plasma membrane integrity (PMI), acrosome integrity (AI<sub>n</sub>) and recovery rate. The obtained data were tested by Z test then presented as mean  $\pm$  standard deviation. The research results from three artificial insemination center showed that motility mean was  $45.00 \pm 3.07\%$ , concentration mean was  $26.09 \pm 3.11 \times 10^6$  cells/0.25mL, longevity mean was  $10.38 \pm 0.75\%$ /hour, PMI mean was  $45.86 \pm 10.67\%$ , AI mean was  $73.99 \pm 5.28\%$  and recovery rate mean was  $64.38 \pm 5.16\%$ . The conclusions of this research were the motility and concentration mean were higher than Indonesian National Standard (INS), longevity mean was lower than Department of Animal Husbandry, Dairying and Fisheries (DADF) standard but PMI and AI<sub>n</sub> mean were higher, the mean value of recovery rate was higher than of proposal value.

*Keywords :buffalo, spermatozoa, post thawing, semen quality*

## INTRODUCTION

Based on Indonesian livestock statistic 2015, buffalo population increased in 2015 (1.381.000 heads) compare to 2014 (1.335.000 heads), yet still lower than 2012 (1.408.000 heads). The national population enhancement is due to the reduction of buffalo slaughtered in 2011-2015 amounted to 15.84%, and it was not because of improvements on the buffalo reproduction efficiency. The population gap between the adult males (13.23%) and adult females (63.01%) is suspected to the low reproductive efficiency in buffalo (Ditjen PKH, 2015). Many steps are taken to address the population gap including artificial insemination programs.

The Indonesian government has set up artificial insemination (AI) centers to produce and distribute frozen bull semen that meet the standards in order to increase the population of buffalo. Post thawing semen evaluation program is a means to identify the suitability of frozen semen produced to standards based on laboratory testing in order to drive the success of AI in the field. Evaluation of post thawing spermatozoa quality was conducted to obtain information on technical parameters affecting spermatozoa fertility after being frozen. The evaluation results were then compared to the standard and proposed values.

Indonesian National Standard (INS) for frozen buffalo semen were based on some values such as progressive motility must be  $\geq 30\%$  and contains  $\geq 25 \times 10^6$  cells/ 0.25 ml (BSN, 2008). Standards for Production of Bovine Frozen Semen provides a reference values for the plasma membrane integrity  $\geq 40\%$ , acrosome integrity  $\geq 70\%$  and the longevity through the incubation test  $\leq 10\%$  every 30 minutes (DADF, 2012). The proposed reference value of the recovery rate is obtained through the approach of fresh semen motility value required in Regulation of the Minister of Agriculture No. 10/2016 that was 70% and post thawing motility value in INS that was 30%. From the comparison of these two values it was obtained the proposed value for recovery rate was 43%.

Based on the reason above, this study was aimed to evaluate and compare the quality of post thawing spermatozoa of buffalo frozen semen produced by artificial insemination centers on standard values and proposed reference value.

## MATERIALS DAN METHODS

This research was conducted at three AI centers, namely Lembang AI Center, South Kalimantan AI Center and North Sumatera AI Center, for six months from December 2016 to May 2017.

### Sample Collection

Materials of this research were 60 samples of buffalo frozen semen that are produced by those three AI centers, each consist of 2 bull and 10, straws per bull, respectively.

### Thawing Frozen Semen

Before evaluation, frozen semen was thawed using warm water (37°C) for 30 seconds. After that, the straw was dried with a tissue, and then the semen was removed and inserted into the eppendorf tube by cutting the manufactory and laboratory plugs on both ends of the straw. The tube was placed in a 37°C water bath for further evaluation.

### Evaluation of Post Thawed Semen Motility

A drop of semen was dropped on the glass objects that have been warmed and then was covered with a glass lid. Motility evaluation was performed using a microscope with a magnification of 400x that taken from five field of views. The percentage motility of spermatozoa was observed with laboran assistance to avoiding subjectivity assessment.

### Evaluation of Semen Concentration

A total of 10  $\mu\text{L}$  of semen mixed in Eppendorf tubes containing formol-saline (990  $\mu\text{L}$ ) were homogenized. Frozen semen samples of 8-10  $\mu\text{L}$  were inserted into the calculated chamber that had been sealed with a cover glass in hemocytometer. The concentration evaluation was done by counting the spermatozoa from five large squares, 4 in the corner and 1 in the middle. The calculation of concentration is using the formula as follows.

$$\text{Number of sperm}/0.25 \text{ mL} = N \times \text{FP} \times 5 \times 0.25 \times 10.000$$

Where:

N : Average number of sperm in A and B chamber

FP : Diluents factors (1:100)

5 : Corrections factor due to only count

five square from 25 square.

0.25 : Corrections factor due to straw size  
0.25 mL.

10.000 : Corrections factor due to cover slip  
depth 0.0001 mL per *chamber*

### Evaluation of Semen Longevity

Spermatozoa longevity is measured by evaluating the motility of spermatozoa incubated at 37°C every 60 minutes from the 0<sup>th</sup> hour to the 4<sup>th</sup> hour.

### Evaluation of Semen Plasma Membrane Integrity (PMI)

Hypo Osmotic Swelling Test (HOST) solution was being made by 0.9 g of fructose and 0.49 g sodium citrate then added by aquades up to 100 ml, then 50 µl frozen semen and 950 µl of HOS solution was homogenized then was incubated at 37°C for 30-45 min. Evaluation of PMI was conducted with a 400x magnification microscope from 10 fields of view to a circular or inflated spermatozoa shape. The calculation of the percentage of PMI was using the following formula:

$$\% \text{ PMI} = \left( \frac{\sum \text{Intact Plasma Membrane of sperm}}{\sum \text{Total sperm}} \right)^{-1} \times 100\%$$

### Evaluation of Semen Acrosome Integrity (AI<sub>n</sub>)

Frozen semen (10µl) and formol-saline solution (990 µL) were homogenized. The observations were performed under a phase contrast microscope with 1000x magnification on 100 different spermatozoa cells. Sperm with a normal and intact acrosome cap was marked by the anterior portion of the head was darker. The calculation of the percentage of acrosome integrity was using the following formula:

$$\% \text{ AI}_n = \left( \frac{\sum \text{Intact Acrosome of sperm}}{\sum \text{Total sperm}} \right)^{-1} \times 100\%$$

### Recovery Rate Evaluation (RR)

Assessment was conducted by comparing the data of post thawing motility with fresh semen motility. The calculation of the percentage of spermatozoa recovery rate was using the following formula.

$$\% \text{ RR} = (\% \text{ Post thawing Motility}) \times (\% \text{ Fresh semen Motility})^{-1} \times 100\%$$

### Statistical Analysis

The obtained data from three insemination centers were tested by Z test then presented as mean ± standard deviation.

## RESULTS AND DISCUSSION

Post thawing semen evaluation was important to assess the success of the freezing process and to maintain the frozen semen standards that were produced by the center before being distributed to buffalo breeders. The success of semen freezing can be seen from the number of successful spermatozoa recovered from the freezing process (Hafez, 2000). The Z test results for the motility, longevity, concentration, recovery rate, PMI and AI<sub>n</sub> values of buffalo spermatozoa post thawing are presented in Table 1.

The motility and concentration mean value were higher than Indonesian National Standard (INS), which were 45.00 ± 3.07%, and 26.09 ± 3.11 × 10<sup>6</sup> cells/0.25ml respectively. The longevity mean value was lower than Department of Animal Husbandry, Dairying and Fisheries (DADF) but plasma membrane integrity (PMI) and acrosome integrity (AI) mean were higher, which were 10.38 ± 0.75%/hour, 45.86 ± 10.67%, and 73.99 ± 5.28%, respectively. The mean value of recovery rate was higher than proposal reference value (PRV), that was 64.38 ± 5.16%.

### Evaluation of Spermatozoa Buffalo Motility

Motility is an important aspect for assessing the fertility of spermatozoa after freezing and thawing to bring sperm through the female reproductive tract to reach the fertilization site (Kumar *et al.*, 2014). The sperm motility depends on Adenosine Tri Phosphates (ATP) results from the oxidative phosphorylation process in the mitochondrial membrane that was transferred to microtubules for motility. Cell damage was caused by cooling and re-heating sperm, consequence from the formation of ice crystals that may damage the structure of lipoproteins in the sperm cell membrane thus affecting the balance of intra and extracellular chemistry potentials as well as the ability to alter the source of energy metabolism for cellular and motion (Herdiawan, 2004).

The mean value of motility obtained was higher than that of Kaka *et al.* (2011) and Ansari *et al.* (2014), each having a post thawing motility value of 43.25 ± 3.40% and 41.67 ± 2.90% in Kundhi buffalo. This difference was thought to be due to differences in buffalo breeds as the source

Table 1. Z Test Results For The Motility, Longevity, Concentration, Recovery Rate, Plasma Membrane Integrity (PMI) And Acrosome Integrity (AIn) Values Of Buffalo Spermatozoa Post Thawing.

Parameters	Values	Types of References		
		INS	DADF	PRV
Motility, %	45.00 ± 3.07 <sup>a</sup>	30.00 <sup>b</sup>	0	0
Concentration, 10 <sup>6</sup> cell/0.25 mL	26.09 ± 3.11 <sup>a</sup>	25.00 <sup>b</sup>	0	0
Longevity, %/hours	10.38 ± 0.75 <sup>a</sup>	0	20.00 <sup>b</sup>	0
PMI, %	45.86 ± 10.67 <sup>a</sup>	0	40.00 <sup>b</sup>	0
AIn, %	73.99 ± 5.28 <sup>a</sup>	0	70.00 <sup>b</sup>	0
Recovery Rate, %	64.38 ± 5.16 <sup>a</sup>	0	0	43.00 <sup>b</sup>

Differences superscript (a,b) in the same row show significant differences (P<0.05); INS = Indonesian National Standard; DADF = Departement of Animal Husbandry, Dairying and Fisheries; PRV = Proposed Reference Values; PMI = Plasma Membrane Integrity; AIn = Acrosome Integrity

of semen and genetic quality of each Bull. Results from previous studies showed that post thawing motility of buffalo semen is higher than that required by INS (30%). Based on that, it should be considered to raise the standard value that listed in INS in order to achieve the increase of buffalo population nationally. Increasing the population is possible because the greater the value of motility post thawing, the greater the probability of fertilization. Septiyani *et al.* (2014) explained that there was a significant relationship between motility with viability ( $r = 0.59$ ;  $p = 0.01$ ) and intact plasma membrane with motility ( $r = 0.69$ ;  $p = 0.01$ ).

#### Evaluation of Spermatozoa Buffalo Concentration

The results obtained from this study indicate that the concentration value in straw produced by AI center is higher than INS. This was because the process of semen packing using a computerized system that allows to adjust the concentration to be filled into the straw at a minimum value of  $25 \times 10^6$  cells/0.25 ml. Even though, manual counting by using Neubraur counting chamber should also be done as a recheck tool to the accuracy of concentration calculating performed by the computer. The calculating accuracy of initial semen volumes, fresh spermatozoa concentrations and volume of dilution were the key success to determining spermatozoa concentrations in straw (0.25 ml) before frozen.

Previous study has been conducted to evaluate the effect of reducing the number of sperm of buffalo to fertility. The results of Andrabi *et al.* (2006) showed that the reduction in the number of the Nili-Ravi buffalo sperm from  $30 \times 10^6$  to  $15 \times 10^6$  cells/ 0.5 mL per insemination dose did not affect the fertility of frozen semen in the field (pregnancy rate 47.19 to 56.78%). The concentration value of Andrabi *et al.* (2006), when converted into straw size 0.25 ml, it would get the value of 15 to  $7.5 \times 10^6$  cells/ 0.25 mL. Gaviraghi *et al.* (2013) reported that the use of 4, 6, and  $8 \times 10^6$  cells/ 0.25 mL concentrations resulted in a not significantly different pregnancy rate, ranging from 47.1 to 49.8%. The results of Andrabi *et al.* (2006) and Gaviraghi *et al.* (2013) were lower than the standards used in Indonesia. This was suspected because in that study, the factors of breeders, inseminators, and acceptor cows of insemination had been well managed.

In general, the frozen semen produced in each center has fulfilled the INS requirements that were more than  $25 \times 10^6$  cells/ 0.25 ml. It showed the ability of each bull to meet the standard of concentration. The determination of the standard value of semen concentration of  $25 \times 10^6$  cells/ 0.25 ml in Indonesia was still relevant to the current condition. It was possible to maintain the feasibility of frozen semen until it was being inseminated to the buffalo breeder. Breeders in Indonesia generally use traditional rearing patterns with a herds system so that the ability to

detect signs of estrus becomes weak and insemination momentum becomes detached. According to Sophian and Gunawan (2015), the degree of success in buffalo insemination should be supported by the condition of the recipients/cow who meet the requirements of good conditions of physical and reproductive, good rearing management, and appropriate use of hormonal preparations.

#### **Evaluation of Spermatozoa Buffalo Longevity**

The result of Z test (Table 1) shows that the obtained longevity value was lower than the DADF value significantly. The results could be interpreted that the survival ability of spermatozoa motility from AI centers was better than the value of DADF and had a great opportunity to do the capacitation and reach the ovum within 4 hours.

Incubation temperature of 37°C is chosen due to it was optimum temperature for spermatozoa metabolism (Kartika *et al.*, 2014). Metabolism rate increases due to the drastic changes on temperature at freezing and thawing. Metabolic rate increases and the life span of spermatozoa decrease as the temperature of semen rise.

Longevities could be prolonged when in a spermatozoa washer solution is added phospholipid in lecithin form. Lecithin had a better tolerance for changes in osmotic pressure in freezing which could damage the structure and function of plasma membranes of spermatozoa (Khalifa and El Saïdy, 2006). The longevity value of this study was better than that reported by Maurya and Tuli (2003) in Murrah buffaloes of 12.17% /h. Rahoo *et al.* (2011) reported that longevity value in Kundhi buffalo was 10.81%/hour. Longevity testing should be performed by the AI Center to evaluate spermatozoa survival abilities in in vitro conditions, judged from its progressive motility at particular temperature and time duration.

#### **Evaluation of Buffalo Spermatozoa Recovery Rate**

The result of Z test (Table 1) showed that the value of obtained RR was higher than the proposed reference value significantly. The high value of RR was thought to be due to the content of lecithin in the diluent. It was able to protect the sperm from the bad effect of freezing by maintaining the normal configuration of the phospholipid layer, which was the main arrangement of spermatozoa cell membrane (Baharun *et al.*, 2017).

Salmani *et al.* (2014) stated that soybean lecithin was more efficient in protecting goat sperm against harmful lipid peroxidation during clotting compared to egg yolks that were containing unsaturated fatty acids. Rizal and Herdis (2010) stated that in the process of oxygen respiration in the mitochondria, saturated fatty acids would react in chains with hydrogen peroxide compounds resulting in new lipid peroxidation reactions. The presence of lipid peroxidation reactions on spermatozoa cell membranes during semen processing was thought to result in contact between semen and oxygen so that oxidative metabolic activity increases and produces an increase in free radical that can decrease life span.

The lipid peroxidation process occurred during the thawing process, so according to Samsudewa and Suryawijaya (2008), the long-term thawing duration would causes the decrease in individual motility to the unusable quality for AI (<40%). According to Lessard *et al.* (2000) which was cited by Sukmawati *et al.* (2014), sperm quality declined after freezing. About 50% of sperm was dead during freezing and sperm that able to survive generally have low fertility.

Based on that, the RR value of buffalo frozen semen that was produced by the AI centers were feasible to use. The frozen semen production process of is came from fresh semen which has an average motility value above 60% with progressive motion ++ or +++, and the result of post thawing motility testing has an average motility value above 40%. Based on the results of the research, RR needs to be assessed and considered as the value of quality standards for frozen semen that were produced by AI Centers.

#### **Evaluation of Membrane Plasma Integrity of Buffalo Spermatozoa**

The result of Z test (Table 1) showed that the obtained PMI value was higher than DADF significantly. The results indicated that the diluents used by the center had an ability to protect the spermatozoa plasma membrane against the adverse effects of low temperatures and high salt concentrations during freezing (Rastegarnia *et al.*, 2013). Common components in diluents that served as a protective against cold shock and as cryoprotectants, were glycerol, egg yolk, milk, low density lipoproteins, and soy lecithin.

The most widely used cryoprotectant in the freezing of semen mammals was glycerol. Glycerol was able to bind water because it had

three hydroxyl groups. Glycerol could diffuse into cells faster, capable of altering large and sharp ice crystals and flexing cell membranes so they were not easily brittle (Gazali and Tambing, 2002). Petrunkina *et al.* (2007) reported that the frozen semen undergoes drastic osmotic changes. Cell experienced dehydrated and shrinkage due to hyper-osmotic pressure during freezing, while thawing cell rehydration occurs due to hypo-osmotic pressure. Hypotonic shock caused water to enter the cell membrane so that the K<sup>+</sup> and Cl<sup>-</sup> intracellular ions came out to lower the concentration gradient and maintained electrical neutrality. The release of the ions lowers intracellular tonicity and caused water out to balance the osmotic pressure in the cell. As a result of the regulation of the volume of the cell visually visible changes to the shape of the tail as a response to changes in osmotic pressure of spermatozoa cells. Spermatozoa with an intact plasma membrane were characterized by a circular or swelling tail (Figure 1).

The results obtained from this study were lower than those reported by Herdis *et al.* (2008) using the AndroMed<sup>®</sup> diluent on the epididymis spermatozoa of Belang buffalo that was  $68.00 \pm 1.10\%$ . However, the results of this study were higher than those reported by Singh *et al.* (2014) in Murrah buffalo using egg yolk tris ( $38.2 \pm 1.31\%$ ) and soybean extract ( $39.2 \pm 1.52\%$ ).

Evaluation of the PMI has not been widely implemented by AI Centers, whereas this evaluation could provide information that the spermatozoa plasma membranes from the frozen semen produced were good. As an indicator of the quality of frozen semen, the PMI showed a significant association with live percent ( $r = 0.62$ ;  $p = 0.003$ ) and motility ( $r = 0.69$ ;  $p = 0.01$ ) (Septiyani *et al.*, 2014).

#### Evaluation of Acrosome Integrity of Buffalo Spermatozoa

The result of Z test (Table 1) shows that the value of Acrosome Integrity obtained was higher than DADF value significantly. These results indicated that diluents used by AI Centers had the ability to protect the integrity of the acrosome cap against temperature changes due to freezing and thawing. Rasul *et al.* (2001) suggested that freezing and thawing processes might caused greater (maximum) damage to acrosome cap, which was characterized by the release of acrosomal enzymes such as hyaluronidase and acrosin, compared with dilution, cooling and equilibration stages.

The presence of a normal and intact acrosome in the spermatozoon were important during fertilization because it contains a hyaluronidase enzyme which had an ability to penetrate into the oocyte pellucid zone and

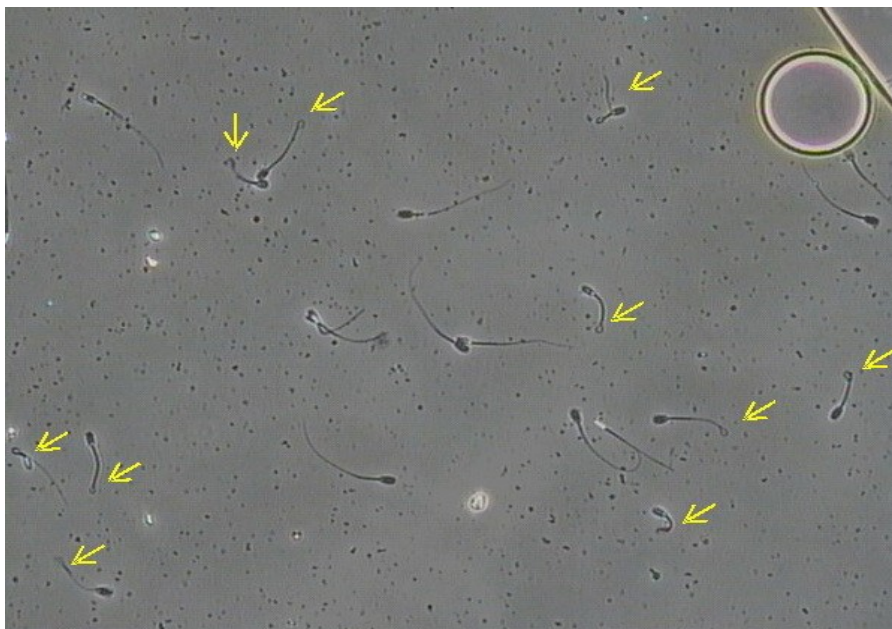


Figure 1. The Positive Reaction of Spermatozoa to the Hypo-Osmotic Swelling Test (HOST) Solution Indicated by a Various Circular Tail (showed by arrows)

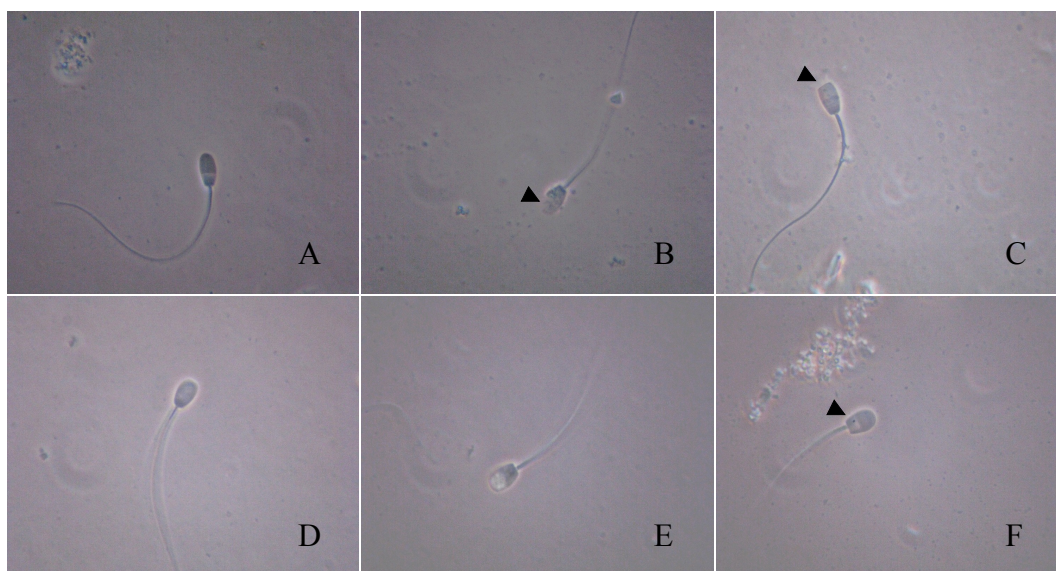


Figure 2. Examples of Some Forms of Acrosome Cap Damage: (A) normal acrosome cap; (B) the spermatozoa head is not smooth; (C) flattening of the apex; (D) the nucleus ring is not visible; (E) anterior is brighter than posterior; and (F) nuclear vacuoles appear as dark dots and can be observe anywhere on the sperm head

increased the opportunity of fertilization (Akhter *et al.*, 2015). The content of lecithin in the diluent was considered more efficient in protecting sperm against lipid peroxidation due to the lower degree of unsaturated fatty acids (Salmani *et al.*, 2014). Wibowo (2014) lipid peroxidation caused the cap of the acrosome to be impaired or damaged, marked by an invisible wrapping line on the head and the nucleus ring and no darker color on the top of the head (Figure 2).

The results obtained from this study were not different from those reported by Chaudhari *et al.* (2015) by evaluating the effectiveness of the tris egg yolk and soybean diluents (Bioxcell® and Optixcell®), with intact acrosome values of 76.83 ± 0.23%; 75.90 ± 0.27% and 78.50 ± 0.25% respectively. Taofik (2012) reported that the integrity of acrosome had the strongest relationship ( $r = 0.917$ ) compared to the progressive motile of sperm ( $r = 0.100$ ) and membrane integrity ( $r = 0.194$ ) to calving rate (CR). The magnitude effect of acrosomes integrity on CR percentage was evidence that the integrity of acrosome contributes to determining the process of zygote development at the beginning of pregnancy. The AI Centers should conduct acrosome integrity testing of each frozen semen product on a regular and scheduled basis.

## CONCLUSIONS

The motility and concentration of sperms mean were higher than Indonesian National Standard (INS), longevity mean was lower than Department of Animal Husbandry, Dairying and Fisheries (DADF) but plasma membrane integrity (PMI) and acrosome integrity (AIn) means were higher. The value of recovery rate was higher than proposal value.

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