Addition of bovine serum albumin (BSA) in cauda epididymal plasma-2 (CEP-2) extender to Ongole grade bull sperm motility and membrane integrity during the freezing process

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Abstract. The semen freezing process causes a decrease in quality, especially sperm motility and membrane damage. Decreasing the semen quality during the freezing process can be prevented by adding semen diluents that fulfill the nutritional needs of sperm, such as CEP-2 and extracellular cryoprotectants, such as BSA. Cauda Epididymal Plasma-2 diluents have a composition like bull plasma cauda epididymis and have been shown to be able to maintain the quality of bull liquid semen at 5ºC for eight days. The addition of BSA with different levels in the CEP-2 extender was expected to support the function of egg yolk in preventing damage to the sperm membrane due to cold shock during the freezing process. The purpose of this study was to determine the best BSA level to maintain sperm motility and membrane integrity during the freezing process. The research material was a three-year-old Ongole grade bull ejaculate which was collected once a week with an artificial vagina and individual motility of at least 70%. The research method was an experimental laboratory with BSA level 0; 0.2; 0.4; 0.6; 0.8 and 1%. The results showed that the highest sperm motility was 0.6% BSA level (42.2±2.58%) and the highest membrane integrity at the BSA level was 0.4% (84.30±2.56%). The study concluded that the addition of BSA in CEP-2 diluents increased the motility and membrane integrity during the freezing process, met the Indonesian National Standards. The research suggested the use of a BSA level of 0.6% in CEP-2 diluents for commercial frozen semen production.

1. Introduction
Artificial Insemination is an application of livestock reproduction biotechnology that supports the increase in population and genetic quality of Indonesian local cattle, such as Ongole grade cattle. One of the factors that support the success of AI was cows inseminated using frozen semen from superior bulls in terms of quality and genetics. High-quality frozen semen should be supported using a diluent that can maintain motility, membrane integrity, and fertility during freezing. The addition of an extender to the ejaculate aims to increase the volume of semen, provide nutrition for sperm, protect against cold shock, buffer, prevent microbial development, maintain membrane isotonic and pH stability [1,2]. The properties of the extender were like the seminal plasma that the motility and integrity of the spermatozoa remain high in frozen storage.

Cauda Epididymal Plasma-2 (CEP-2) was a type of semen extender that has a chemical composition and osmolarity like seminal plasma in the cauda epididymis. The composition of CEP-2 was NaCl, KCl, CaCl₂·(H₂O)₉, NaH₂PO₄, KH₂PO₄, fructose, sorbitol, tris, gentamicin, citric acid [3]. Cauda Epididymal
Plasma-2 extender had been shown to maintain the quality of liquid semen stored at 5°C and has not been widely used for frozen semen processing. Frozen storage using liquid nitrogen (-196°C) caused a decrease in sperm motility and mortality of more than 50% due to membrane damage [4]. The addition of extracellular cryoprotectants, such as Bovine Serum Albumin (BSA), was expected to support the function of egg yolk in maintaining membrane integrity, so that sperm motility and fertility could be maintained during frozen storage. The use of BSA in semen extender had been shown to maintain sperm motility, plasma membrane integrity, and acrosome against changes in temperature during freezing and thawing. This helped sperm to survive in the female reproductive tract [5]. A study of BSA supplementation in BIOXcell® extender (IMV Technologies, L’Agile, France) reported that the viability of Piedmontese bull sperm was (38.743%±0.854) after storage at 4°C for 31 days with 8 mg ml BSA level. The BSA levels used were 0, 1, 4, 8, 12, and 16 mg/ml [6]. Supplementation of BSA with different levels in CEP-2 diluent has not been widely studied, especially the motility and integrity of Ongole grade sperm during frozen semen processing.

2. Materials and methods

2.1. Materials
This study used a three-year-old Ongole grade bull ejaculate reared in Beef Cattle Research Station, Grati Sub district, Pasuruan Regency, East Java Province, Indonesia. Ejaculate collection was done once a week. The feed given was concentrates (Sukamakmur KUTT, Grati District, Pasuruan Regency), Indigofera spicata, elephant grass, straw, and water were given in ad libitum. Ejaculate for frozen semen processing has individual motility> 70%, mass motility >2+, sperm concentration 1000 million cells/mL, viability> 80%, and abnormality <15% [7].

2.2. Methods

2.2.1. Ejaculate collection
The entire bull stomach and preputium were cleaned before the ejaculate collection to prevent a decrease in semen quality. Semen collection using the artificial vaginal method.

2.2.2. Sperm motility assessment

2.2.2.1 Sperm mass motility. The mass motility assessed the movement of the sperm group. The ejaculate was dropped on a glass object without covering the glass cover and observing it under a microscope with 100× of magnification. Assessment of mass motility was sperm moving actively and very fast like a dark cloud when it was going to rain (+++), sperm moving not too fast and looking like dark clouds (++), sperm visible only individual movement and no group of sperm (+) and immobile sperm (0) [8,9].

2.2.2.2 Individual motility. Assessment of individual motility based on individual sperm motion. Ejaculate or treated semen was dripped on the object-glass, covered with a cover glass, and observed under a microscope with 400× magnification. The individual motility was based on the number of sperm moving progressively forward (backward and circular movements were not counted) compared to sperm that were stationary in five different visual fields. Assessment of individual motility in the percentage of mobile sperm [10].

2.2.3. Dilution of semen. The ejaculate used for frozen semen was 3 mL and divided into six treatments of 0.5 mL each. The treatment used CEP-2 + 10% egg yolk extender with different BSA levels, i.e., 0; 0.2; 0.4; 0.6; 0.8 and 1%. The addition of diluent to the ejaculate was worked at 37°C (1:1) and semen was placed in a 15% volume glass tube immersed in water (water jacket) to reduce the temperature. The semen was added to the extender gradually at a temperature of 30; 25; 20 and 12°C (A2). The semen
was put into the cool top, that the temperature dropped to 5°C and if the semen temperature was stable,
then the semen was added with an extender containing intracellular cryoprotectant (7% glycerol) [11].
The determination of sperm individual motility and membrane integrity were done on semen reaching
a temperature of 12°C [12].

2.2.4. Equilibration. Equilibration time is the time required for sperm to adjust to an extender containing
cryoprotectant at 5°C. The length of equilibration time varies between 4–22 hours and this study used
four hours to prevent membrane damage [11]. The sperm individual motility and membrane integrity
were observed after equilibration time.

2.2.5. Filling and sealing straw. Semen storage using a mini straw volume of 0.25 mL was equivalent
to 25 million sperm. The semen was put into straw utilizing an automatic filling and sealing machine in
a cool top (5°C) [11].

2.2.6. Freezing. Adjusting the semen temperature in the straw towards the freezing process, then the
straw was evaporated over liquid nitrogen (-140°C) for nine minutes to prevent sperm death due to
drastic temperature changes. Straws were arranged in a canister and soaked in liquid nitrogen (-196°C)
to extend the semen shelf life until they were ready to be inseminated to cows[13,14].

2.2.7. Post thawing motility. Straws had stored in containers filled with liquid nitrogen (-196°C) for at
least 24 hours. Observing of individual motility and sperm membrane integrity (post-thawing) was
carried out after freezing [11].

2.2.8. Hyperosmotic swelling test. A 500 μL of the hypo-osmotic solution was added to 50 μL of
ejaculate or treated semen. The solution was incubated at 37°C in a water bath for 45 minutes. Then 10
μL of the solution was placed on the object-glass and observed under a 400× magnification light
microscope. Observations of membrane integrity include: the tip of the tail was circular or there was
swelling of the head indicating that the integrity of the sperm membrane was good (positive HOST) and
sperm with a straight tail indicated damaged membrane integrity [14] (see figure 1). The calculation was
carried out on 200 sperm to minimize errors and the ratio was expressed in percentage units.

2.3. Observation variables
Sperm motility and membrane integrity with the best BSA levels during frozen semen processing (after
dilution, before freezing, and post-thawing).

2.4. Data analysis
Statistical analysis was performed using R Studio version 3.6.1 (USA) with a significance level of 5%.
Comparison of the effect of different BSA levels on sperm individual motility and membrane integrity
in CEP-2 diluent was analyzed using ANOVA repeated measured or time series methods followed by
Tukey's test if the data met the norms of normality and homogeneity. If the assumptions were not met,
the comparison test was analyzed by the Kruskal Walls method followed by the Wilcoxon test.

3. Results and discussion

3.1. Ejaculate motility
The results showed that the ejaculate mass motility was 3+, which was indicated by a rapid movement
of sperm such as a black cloud wave that would rain. This value indicated that the mass motility in the
normal range of bull semen was between 2+ and 3+ [15]. The percentage of individual motility was an
important quality indicator to determine the ejaculate for frozen semen processing. The sperm motility
of the study results was 75% and this result met the requirement of the individual motility of the
Indonesian National Standard (> 70%). Generally, bull sperm has individual motility of 40–75% [16].
3.2. Sperm motility during the freezing process

The percentage of individual sperm motility decreased along with the freezing process (see table 1). Sperms used the nutrients in the diluent to maintain motility and membrane integrity during the freezing process. The cryopreservation process affected the morphological and ultrastructure changes of the sperm which caused damage to the cytosolic and nuclear membranes. Most of the damage to sperm that occurred during freezing was associated with intracellular ice crystallization in a decrease in temperature towards freezing and/or thawing at water bath temperature [17]. The addition of intracellular cryoprotectants caused an increase in the extender osmotic pressure and had the potential to damage sperm. Water would leave the cell causing the sperm to shrink, then sperm to swell when cryoprotectant and water entered back into the cell to maintain the chemical balance and slowly return to the isosmotic when the water left the cell. Changes in osmotic pressure resulted in a significant loss of sperm functional integrity, such as motility, or even cell death without loss of plasma membrane integrity [18]. Glycerol is a type of cryoprotectant that is widely used for cell coagulation, including mammalian sperm [19]. Too high a glycerol concentration in a cement diluent impressed the osmotic pressure which resulted in sperm metabolism and toxic to the plasma membrane causing cell death. The 7% glycerol content in basic diluent was the best concentration to maintain motility (45.83±4.91%) and membrane integrity (57.83±2.17%) of buffalo sperm during the freezing process [18].

| BSA Level (%) | After diluting (%) | Freezing process (%) | Post thawing motility (%) |
|---------------|--------------------|----------------------|--------------------------|
|               | Motility HOST (+)  | Before freezing (%)  | Post thawing motility HOST (+) |
| 0             | 69±2.11<sup>a</sup> | 75.13±9.83<sup>a</sup> | 63±2.58<sup>b</sup> | 59.99±3.21<sup>a</sup> | 41±2.11<sup>a</sup> | 78.75±4.84<sup>a</sup> |
| 0.2           | 69±2.11<sup>a</sup> | 86.44±2.03<sup>ab</sup> | 63±2.58<sup>a</sup> | 85.92±5.33<sup>a</sup> | 41±2.11<sup>a</sup> | 76.26±6.06<sup>c</sup> |
| 0.4           | 69±2.11<sup>a</sup> | 88.70±4.74<sup>a</sup> | 63±2.58<sup>a</sup> | 86.06±4.03<sup>a</sup> | 41±2.11<sup>a</sup> | 84.30±2.56<sup>a</sup> |
| 0.6           | 69±2.11<sup>a</sup> | 83.03±1.43<sup>b</sup> | 63±2.58<sup>a</sup> | 81.56±3.31<sup>b</sup> | 42±2.58<sup>b</sup> | 74.55±3.30<sup>a</sup> |
| 0.8           | 69±2.11<sup>a</sup> | 89.65±2.49<sup>a</sup> | 63±2.58<sup>a</sup> | 84.37±3.72<sup>ab</sup> | 41±2.11<sup>a</sup> | 75.61±5.51<sup>b</sup> |
| 1             | 69±2.11<sup>a</sup> | 87.84±3.92<sup>a</sup> | 63±2.58<sup>a</sup> | 83.83±3.31<sup>ab</sup> | 41±2.11<sup>a</sup> | 76.90±3.71<sup>b</sup> |

Table 1. Sperm motility and membrane integrity during the freezing process.

The individual motility using different BSA levels after freezing was more than 40%. These results met the Indonesian national standard that the frozen semen requirement inseminated in cows has motility of at least 40% to support AI. The results showed that the BSA level had not impressed sperm motility decrease during freezing. Levels 0.6% (42±2.58%) was the best proportion of individual motility after freezing. Commercial frozen semen should use a BSA level of 0.6% for efficiency costs production. The cause of individual motility was decreased freezing due to sperm loss of aggressive and progressive potential with releasing of the aspartate-aminotransferase (AspAT) enzyme to the plasma membrane, then ATP products stopped resulting in sperm death [20].

The CEP-2 and 10% egg yolk had been maintained the semen quality stored at 5°C for six days [3] and egg yolk as an extracellular cryoprotectant in semen for sperm survival in cold and frozen storage. Egg yolks contain phospholipids, a Low-Density Lipoprotein (LDL) fraction, which had a component that effectively protected the sperm membrane from temperature changes and cold shock during the freezing process. The addition of egg yolk to the extender serves to maintain the stability of the integrity and the lipoprotein membrane layer [21]. The function of egg yolk was supported by the addition of BSA as an extracellular cryoprotectant and antioxidant in CEP-2, substituting for a decrease in the concentration of various ingredients contained in semen plasma during the dilution process to freezing, thus providing maximum protection for the stability of the sperm membrane during the freezing process [22]. Bovine Serum Albumin bonded to the plasma membrane and phospholipid groups that bonded to proteins and glycoproteins, that membrane particle accumulated due to the influence of changes in temperature drop and cold shock during the freezing process [23].
3.3. Membrane integrity
The results showed that sperm membrane integrity decreased with different stages of the freezing process and BSA levels in CEP-2 (P<0.05). The highest sperm integrity post-thawing at a BSA level of 0.4% was 84.30±2.56% (see table 1 and figure 1). This was assumed by the nutrients in the diluent needed by sperm to maintain permeability and membrane integrity which was closely related to the overall quality of semen. CEP-2+10% egg yolk was optimal in maintaining permeability and sperm membrane integrity during the frozen semen process.

Figure 1. Observations of post-thawing membrane integrity using a light microscope (CX-21, Olympus, Japan). The intact membrane with a tail coiled or curled up (a), an incomplete membrane with a straight tail (b)

Egg yolk in semen diluent acts as an energy source and protective agent. The protective agents of egg yolk are lipoproteins, especially phospholipids, which are identified as effective components for protecting the integrity of the sperm membrane. Lipoprotein and lecithin in egg yolk can prevent the flow of Ca²⁺ ions into cells which can damage the sperm membrane. The egg yolk in the diluent acts as an extracellular cryoprotectant that protects sperm acrosome during the freezing process[14,24]. Bovine Serum Albumin supplementation in semen extender was known to remove free radicals from the reactive oxidative species (ROS) and to protect sperm membrane integrity from lipid peroxidation reactions during the cryopreservation process. The concentration of BSA was 20 mg/mL in semen extender of Akkaraman ram increased post-thawing motility (51.2%) and viability (78.2%), and protected membrane integrity from morphological abnormalities (11.8%) and damage acrosome (3.6%) [25].

4. Conclusion
The highest post-thawing individual motility was at a BSA level of 0.6% (42±2.58%) above the individual sperm motility value according to the Indonesian national standard for AI application and a level of 0.6% was recommended for use as an extracellular cryoprotectant in commercial frozen semen production. The highest post-thawing membrane integrity was at the BSA level of 0.4% (84.30±2.56%).

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