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Effect of Different Centrifugation duration on *Simmental* Bull Sperm Quality and Membrane Status after Sexing, Cooling and Freezing Processes

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ABSTRACT

The Percoll density-gradient centrifugation was the most widely used method that able to affect sperm quality during the separation of X and Y spermatozoa. Sexing using Percoll density-gradient centrifugation method at 850 x g for 5 min could afford 85% of X and Y sperm separation. However, the technical problem would appear for 5 min centrifugation in which sperms at the under layer population were not able to obtain the tube base and led to less X and Y sperm separation. Whereas the treatment for 7 min centrifugation had a high level separation power as sperms were able to reach the tube base. The study examines the effect of different duration of Percoll density-gradient centrifugation method on the quality of spermatozoa after sexing, before freezing, and post thawing processes. Fresh semen sample were routinely collected twice a week from two *Simmental* bulls aged 4-9 years using artificial vagina at Ungaran artificial insemination (AI) center, Central Java, Indonesia. The sperm which had individual motility more than 70% were selected for the freezing process. Sexing sperm using Percoll density-gradient centrifugation method for 5 and 7 min was applied in this study. Sperm quality after sexing, before freezing, and post thawing processes was assessed under a light microscope at 400 x magnification and its concentration was evaluated using haemocytometer. Data were analyzed by a pair of t-test. For all statistical analyses, the level of significance was $P<0.05$. There were significant differences ($P<0.05$) on sperm qualities among 5 and 7 min of centrifugation treatments. The study demonstrated that 5 min centrifugation treatment using Percoll gradient provided significantly better sperm motility, viability and concentration compared to 7 min centrifugation treatment ($P<0.05$). Furthermore, a lower number of abnormal sperms were also found significantly at 5 min centrifugation treatment. Our findings suggest that 5 min would be a good and acceptable duration to centrifuge *Simmental* bull sperms using Percoll density-gradient.

Keywords: Percoll density-gradient centrifugation; sperm motility; viability; abnormality; sperm concentration

INTRODUCTION

Sexed semen plays crucial role to produce the desired gender offspring. One great example would be the production of males for meat production [17]. Also, determination of the bovine offspring sex is very important for cattle genetic improvement [6]. Offspring from animal species have been produced from artificial insemination or in vitro fertilization using sperm that has been sorted into X- and Y-chromosome-bearing populations [18]. X and Y spermatozoa can be separated using sorting methods such as flow cytometry, albumin sedimentation, and Percoll density-gradient centrifugation [13, 25].

The separation of bovine X- or Y-chromosome bearing spermatozoa (X or Y sperm) has been successfully obtained through centrifugation of spermatozoa on two Percoll discontinuous density gradients. For example, [24] centrifuged cattle sperm in two Percoll discontinuous gradients consisting 10 layers of 0.6 ml Percoll solution with densities ranging from 1.034 to 1.068 g/ml. This method provided an enrichment of over 75 and 65% of X or Y sperm fractions respectively as verified by in situ hybridization. A further study which used the same methodology performed by [9] found that sperm from the top and bottom fraction resulted 75 and 92% of male and female embryo respectively.

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Percoll density-gradient centrifugation method could be used to assess economic value and led to greater ease of use [27]. In addition, Percoll medium is also made with various density, low viscosity, and not poisonous, and it hasn't pierced into cell membrane. Moreover, it can be separated, has no negative effect on spermatozoa separation, and is easier in releasing from substance as well as in creating gradient on centrifugation. Sexing using Percoll density-gradient centrifugation method at 850 x g for 5 min could afford 85% of X and Y sperm separation which was identified based on the length and the head of spermatozoa [26]. However, the technical problem appeared for 5 min centrifugation in which sperms at the under layer population were not able to obtain the tube base and led to less X and Y sperm separation. Whereas the treatment for 7 min centrifugation had a high level separation power as sperms were able to reach the tube base.

Objectives:

This research aimed at evaluating the influence of different centrifugation duration on sperm quality in terms of motility, viability, abnormality and concentration after sexing, before freezing, and post thawing.

Materials and Methods

3.1. Semen source:

Fresh semen sample were routinely collected twice a week from two *Simmental* bulls aged 4-9 years using artificial vagina at Ungaran artificial insemination (AI) center, Central Java, Indonesia. The sperm which had individual motility more than 70% were selected for the freezing process.

3.2. Percoll gradient:

The preparation of a discontinuous density-gradient was explained as follow: Percoll (Sigma, Singapore) was mixed 1:4 with Andromed @ (Germany) extender diluted with aquades. Andromed extender was generated into several concentrations such as 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60% and 65%. A Percoll discontinuous density-gradient was layered in a 15-ml polystyrene centrifuge tube, comprised from high (65%) to low (20%) concentrations. Each concentration consisted of 0.5 ml or in a total volume of 5 ml [26].

3.3. Experimental design:

Sexing using Percoll density-gradient centrifugation method for 5 and 7 min was applied in this research. Both the top (Y sperm) and under fraction (X sperm) were taken and frozen at -179^{o}C for 24 hours and then thawed, replicated 10 times. It referred to separation process based on the different number of DNA located on the head of spermatozoa. Similar to [13], the basic principle of separation through centrifugation was based on the density differences.

3.4. Sexing sperm (separation X and Y sperm):

One ml semen was placed in a tube containing Percoll gradient concentration and centrifuged at 850 x g for 5 and 7 min. Two ml liquid from the centrifugation result was taken from the bottom fraction (X sperm population) as well as from the top fraction (Y sperm population). Moreover, they were washed in 3 ml of Andromed and centrifuged at 550 x g for 5 min. After centrifugation, the supernatant was carefully removed. Then, bottom fraction (2 ml) of sorted semen which contained more spermatozoa was taken [26]. Finally, sperm qualities were tested on the basis of motility (%), viability (%), abnormality (%), concentration, and sperm membrane structure under Scanning Electron Microscope (SEM).

3.5. Processing of post sexing sperm:

Two ml liquid was taken from the top and the bottom fraction and each of them was added with 2 ml of Andromed extender. Then, these liquid were put in a tube containing warm water (30^{o}C) and were placed in cool tube (4^{o}C). After that we waited until the temperature decrease to 5^{o}C. Diluted semen was inserted into straw (0.25 ml), and evaluated using Scanning Electron Microscope. Straw was equilibrated by putting it in liquid nitrogen steam for 10 min, and then put it in liquid nitrogen at -179^{o}C. After 24 hours, straw was thawed by placing it in warm water (47^{o}C) for 7 min [16]. Finally, motility, viability, abnormality, and sperm concentration were evaluated.

3.6. Assessments of sperm motility and concentration:

Sperm motility assessment was subjectively determined by dropping semen in a glass object covered with a cover glass, and observed under a light microscope at 400 x magnification. One of 200 progressive spermatozoa who moved forward was considered as motile spermatozoa and the results were presented as percentages [7, 14, 10, 8]. Hemocytometer was used to observe sperm concentration. The number of spermatozoa was manually calculated using light microscope at 400 x magnification [7].

3.7. Assessments of sperm viability and abnormalities:

The percentage of sperm viability was carried out individually, on a drop of semen placed in a glass object and then mixed with negrosin eosin. Moreover, this mixture was subsequently examined under a light microscope at 400 x magnification. Dead sperms were indicated by red color, while live sperm had no color as shown in Fig. 1.

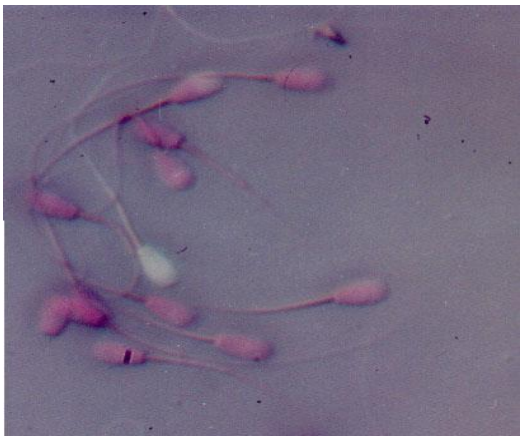

Fig. 1: Live (no color) and dead (red) sperms under a light microscope with 400 x magnification.

3.8. Sperm preparation procedure for SEM analysis:

The media as well as the semen preparation procedure was modified from the Laboratory of Scanning Electron Microscopy [19]. In fixation phase, sample was immersed in 3% of glutaraldehyde buffered with 0.1 M of phosphate at room temperature for 90 min. After that, sample was washed three times in 0.1 M of phosphate buffer for 15 min. In post fixation phase, sample was immersed in 2% of osmium tetroxide with 0.1 M of phosphate buffer and placed in a light tight container for 90 min. The pH was adjusted to 7.2 for 2-4 hours at room temperature. Furthermore, sample was washed three times in 0.1 M of phosphate buffer for 15 min. The pH was set to 7.2. Moreover, sample was dehydrated with a graded ethanol solution in water at 30%, 50%, 70%, 80%, 90%, 95%, and 100% for 10 min of each followed by critical point drying, sample mounting and metal coating processes. In the final phase, specimens were viewed under Scanning Electron Microscope (SEM).

A 0.2 M phosphate buffer consisted of solution A containing 35.82 g/500 ml $Na_2HPO_4.12H_2O$ or 17.8 g/500 ml $Na_2HPO_4.2H_2O$ and solution B containing 15.6 g/500 ml $NaH_2PO_4.2H_2O$ or 13.6 g/500 ml $NaH_2PO_4.H_2O$. Moreover, solution A 36 ml, solution B 14 ml and distilled water 50 ml were added to prepare 0.1 M phosphate buffer with pH 7.2. Whereas, 0.2 M phosphate buffer50 ml, 25% glutaraldehyde 12 ml and distilled water 38 ml were added to prepare 3% glutaraldehyde.

3.9. Data analysis:

The research applied a factorial experiment with randomized group design. Data were analyzed by a pair of t-test. For all statistical analyses, the level of significance was $P<0.05$.

Results:

Sperm motility, viability and abnormality:

A comparison of data on sperm motility at the top and the bottom layers after sexing, cooling and freezing processes is presented in Table 1. The percentage of motile sperms at the top and the bottom layers after sexing, cooling and post thawing processes differed significantly ($P<0.05$) among 5 and 7 min of centrifugation treatments. Overall motility specifically at the upper layer in those three different processes was significantly higher ($P<0.05$) when it was centrifuged for 5 min than that of 7 min. However, unless in post thawing process the percentage of motile sperms at the bottom layer after sexing and cooling processes was significantly lower ($P<0.05$) when it was centrifuged for 5 min than that of 7 min.

Table 1: Sperm motility after sexing, cooling and freezing processes (%).

Treatment	After sexing		After cooling at 5°C		Post thawing	
	Top	Bottom	Top	Bottom	Top	Bottom
5 min	61±15.42[a]	67.5±7.55[a]	49.5±14.62[a]	56,5±13.55[a]	36,5±13.13[a]	39±9.94[a]
7 min	57±12.52[b]	68±2.58[b]	47.5±11.07[b]	60±5.27[b]	34±9.66[b]	38.5±6.69[b]

Values are mean±SD. [a, b] Values in a column with different superscript differ ($P<0.05$).

The percentages of sperm viability at the top and the bottom layers after sexing, cooling and freezing processes are shown in Table 2. Comparing the centrifugation results of sperm viability at the top and the bottom layers for 5 min and that of 7 min in those three different processes the study found that there were significant differences ($P<0.05$) between them. Moreover, it was generally found in all processes that the percentage of viable sperms at the bottom layer in particular, was significantly higher ($P<0.05$) when it was centrifuged for 7 min than of 5 min. Nevertheless, unless after cooling process viable sperms at the top layer after sexing and post thawing treatments was significantly lower ($P<0.05$)

when it was centrifuged for 7 min than that of 5 min.

Table 2: Sperm viability after sexing, cooling and freezing processes (%).

Treatment	After sexing		After cooling at 5°C		Post thawing	
	Top	Bottom	Top	Bottom	Top	Bottom
5 min	75,27±9,95[a]	79,68±5,35[a]	61,65±7,51[a]	69,92±6,11[a]	61,01±5,75[a]	60,43±4,15[a]
7 min	71,17±7,95[b]	80,04±5,18[b]	67,37±5,93[b]	70,99±4,39[b]	57,46±4,82[b]	63,91±3,82[b]

Within a column, means with different superscript [(a, b)] differed (P<0.05).

Data on sperm abnormality at the top and the bottom layers after sexing, cooling and freezing processes are presented in Table 3. In those three different processes it was found out that there were significant differences (P<0.05) on the percentage of abnormal sperms at the top and the bottom layers between 5 and 7 min of centrifugation treatments. In addition, the number of abnormal sperms in those three different processes, particularly at the upper layer was significantly lower (P<0.05) when it was centrifuged for 5 min than that of 7 min. However, unless after sexing process 5 min centrifugation treatment provided significantly a higher abnormal sperms (P<0.05) at the bottom layer after cooling and post thawing processes compared to 7 min centrifugation treatment.

Table 3. Sperm abnormality after sexing, cooling and freezing processes (%).

Treatment	After sexing		After cooling at 5°C		Post thawing	
	Top	Bottom	Top	Bottom	Top	Bottom
5 min	17,64±2,06[a]	17,10±1,12[a]	18,34±1,05[a]	18,40±1,14[a]	19,35±1,19[a]	19,45±0,52[a]
7 min	18,59±1,35[b]	17,17±0,69[b]	19,53±0,61[b]	18,28±0,07[b]	19,91±0,52[b]	18,84±0,47[b]

[a, b] Means with different superscript within a column differ (P<0.05).

Sperms concentration:

Comparing the results of sperm concentration at the top and the bottom layers after freezing processes the study discovered that there were significant differences (P<0.05) among 5 and 7 min centrifugation treatments as shown in Table 4. After centrifugation for 5 min, the sperm concentration as the top layer was significantly higher (P<0.05) but it became significantly lower (P<0.05) at the bottom layer than those which was centrifuged for 7 min.

Table 4: Spermatozoa concentration after freezing.

Treatment	Post thawing	
	Top (10^6)	Bottom (10^6)
5 min	457±144.76[a]	482±98.75[a]
7 min	445+133.10[b]	572+71.46[b]

[a, b] Means with different superscript within a column differ (P<0.05).

Analysis of sperm membrane:

Fig. 2 shows sperms in various conditions. Firstly, the figures show that motile sperms have a good membrane as shown in Fig. 2A and B. Secondly, looking at Fig. 2 C and D it was found that the frozen-thawed spermatozoa without sexing process were not intact between the sperm's head and its neck. Meanwhile Fig. 2 E and F demonstrate sperms conditions after centrifugation treatment and freezing process. The last figure shows sperm with a folded tail.

Discussion:

The preliminary tests showed that fresh semen used in the experiment had good quality. It was found 80% of motile sperms and 6.52% of abnormal sperms. Sperm quality that was beyond the minimal standard for male cattle (having more than 500 million/ml of spermatozoa, more than 50% of progressive movement, and 80% of normal morphology or abnormal sperms less than 20%) could be considered normal [7]. By considering these results, the experiments could be continued to the next processes.

Particles with different weight, size and shape will precipitate with different velocity. Bottom layers have a high density of X sperms, whereas the top layers have a low density of Y sperms. Y sperms have smaller head, lighter, faster, and have more forward movement. On the other hand, X sperms create more residues. As discovered by [26], sperm separation treatment using a 10-Percoll density-gradient centrifugation for 5 min was able to separate 85% of spermatozoa (Y sperms at the top layer and X sperms at the bottom layer). Motile sperms have sharply decreased after treated by centrifugation and freezing process. These treatments led to the detrimental effect on sperm membrane and therefore dropped the quality of spermatozoa incisively.

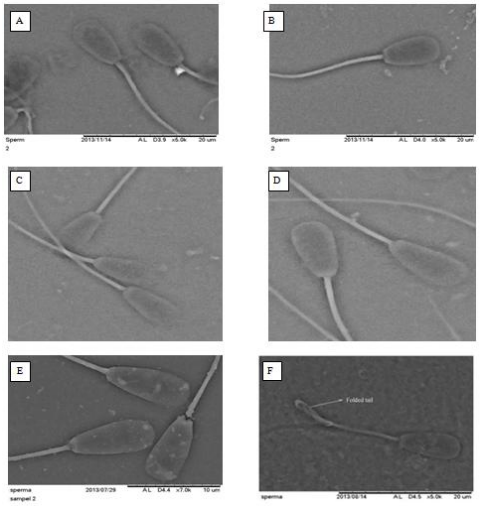

Fig. 2: Spermatozoa observations with Scanning Electron Microscope (SEM). A and B =Fresh sperms with good intact membranes between the sperm's head and its tail; C and D = Frozen-thawed sperms; E and F = Sperms conditions after centrifugation treatment and freezing process.

Sperm motility, viability and abnormality:

As shown in Table 1, the centrifugation treatment for 7 min significantly declined the number of motile sperms at the top layers than that of 5 min. This is because the longer the duration of the centrifugation the more damage the sperm membrane. Furthermore, sperm quality will decline as an increase in the number of broken membrane. As mentioned by [16], freezing process was able to damage sperm membrane and dropped sperm motility. Similarly, [26] found that centrifugation treatment affected sperm membrane structure and function, and broke the parts between the sperm head and tail.

The Indonesian National Standard requires 40% of motile sperms in post thawing process for frozen semen. The study found that 7 min centrifugation treatment provided 40% of motile sperms at bottom layer and 30% - 40% of motile sperms at the top layer. It means that the semen was eligible for artificial insemination use. [22] mentioned that centrifugation procedure did not interfere with the cleavage rate during in vitro embryo production, but low rate of blastocysts was seen in Percoll gradient.

With regards to sperm viability, the study observed that the number of viable sperms after sexing, cooling and freezing process was still good. The extender was able to maintain viability because it did not contain egg yolk that cause agglutination in bovine semen.[4] found bull frozen-thawed sperms which were removed from egg yolk extender showed similar viability result to fresh semen. The agglutination also occurs during the artificial insemination process.

Another results found that abnormal sperms were indicated by folded tailor broken sperm's head. The sperm abnormality might be caused by the centrifugation process and cold shock although we used Percoll gradient and good diluents. This result is not in agreement with [1] who found that Andromed vegetable lecithin from soybeans could protect the sperm membrane from the cold shock. Andromed diluents have capabilities to minimize abnormal spermatozoa by protecting sperm membrane during the sexing process. Andromed have osmolarity 330 and 320 mOsm [20]. According to [12], membrane integrity and sperm motility were able to be obtained best when the number of osmolarity diluter was about 300 mOsm.

Sperms concentration:

Another result found that fresh semen concentration was 1,052.50 x 10^6, and it was considered as normal category. In daily basis, bulls can produce approximately 3.2–6.7 (10^9) spermatozoa. However, [16] argued that about 1,062±124 million of spermatozoa could be collected using artificial vagina. Moreover, [7] discovered that a young bull was able to produce about 2 x 10^8 sperm/ml, while a mature bull can produce up to 1.8 x 10^9 sperm/ml.

Analysis of sperm membrane:

The quality of sperm membrane relates to sperm motility and viability. Therefore, bad quality membrane will affect sperm motility and viability. Figure 2 F clearly demonstrates a folded sperm's tail. This suggests that centrifugation and freezing processes might also damage sperm membrane, thus causing a decrease on sperm motility and viability. In addition, centrifugation process provided free radicals namely reactive oxygen species (ROS) that damaged the sperm membrane. ROS are free radicals that play a crucial role in many sperm physiological processes such as capacitating, hyper activation and sperm-oocytes fusion [3, 5].

Sperm sorting procedures force sperm cells to a set of stressful steps that can trigger an increase of

ROS production and consequently reduce sorted semen quality. Antioxidant EGCG and SOD in association with seminal plasma are effective in exerting some compensatory protection against the detrimental effect of sorted semen storage while their action is not evident during the sorting procedures [28]. Spermatozoa are highly sensitive and will be damage if they have a high concentration of ROS [2]. The higher the ROS concentration the more lipids per oxidation can be produced and then will have higher risk to sperm membrane damage, interrupted abnormal metabolism morphology, decrease motility and finally produced low sperm fertility [11]. Lipid per oxidation is a trigger for the damage of membrane plasma integrity which affects cell membrane permeability, enzyme inactivation, DNA structural damage and survival spermatozoa [15]. Physical influence on spermatozoa, for example through freezing process could lead to the relocation of several proteins as proteins GLUT3 [23]. Furthermore, ultra structure condition of spermatozoa can influence fertilization [21].

Conclusion:

The study demonstrated that 5 min centrifugation treatment using Percoll gradient provided significantly better sperm motility, viability and concentration compared to 7 min centrifugation treatment. Furthermore, a lower number of abnormal sperms were also found at 5 min centrifugation treatment. Our findings suggest that 5 min is an acceptable duration to centrifuge *Simmental* bull sperms using Percoll density-gradient.

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Authors'Contribution:

Prof. Trinil Susilawati developed the idea and wrote the manuscript. Dr. Sri Rahayu was responsible for laboratory assistances especially for Scanning Electron Microscope (SEM) analysis, and Dr. Setyabudi Udrayana was person in charge for sexing processes. Dr. Herni Sudarwati performed the statistical analysis. Eko Nugroho was responsible for editing the manuscript and submitting the full paper.

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There is no conflict of interest.

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