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*Original Article*

# The Effect of Incubation Time on the Quality of Post-Thawed Ram Sexed Sperm

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**Abstract**

**Objective:** The sperm sexing using an albumin column requires an incubation process at a certain temperature for sperm to move through the albumin layer. It is thought that the incubation time can influence the quality of the semen resulting from post-thawing sexing. The aim of this research was to determine the effect of incubation time on the quality of sexed sperm post-thawing and to find out the optimum incubation time that provide the best sperm quality.
**Methods:** This research was carried out in a completely randomized design, using three incubation times (T1= 45 minutes, T2= 60 minutes, T3= 75 minutes) with six replications. The parameters consisted of motility, abnormality and acrosome integrity of sexed sperm in the upper and bottom fractions. Data were analyzed using analysis of variance (Anova) and continued with the Duncan test.
**Results:** The results showed that the incubation time had a significant effect on motility, intact acrosome cap and recovery rate, but had no significant effect on abnormality of post thawed sexed sperm. For both the upper and bottom fraction, incubation times of 45 and 60 minutes produced the best motility and recovery rate compared to 75 minutes, but the best intact acrosome cap was obtained from an incubation time of 45 minutes.
**Conclusions:** Based on the research results, it can be concluded that the length of incubation time influences the quality of post thawed ram sexed sperm, and 45 minutes is the optimal incubation time to provide the best quality.

**Keywords:** Incubation time; Sperm sexing; Ram

## INTRODUCTION

The use of sperm sexing technology has become an appropriate alternative in the application of artificial insemination (AI). AI has been widely applied to large livestock, but only to a small extent to small ruminant such as sheep and goats. The presence of sperm sexing technology is expected to expand the application of AI to small livestock, especially to local sheep with the aim of reproductive efficiency. However, many researches need to be done to obtain sufficient sexed sperm for artificial insemination

Various sperm sexing methods have been applied, and the flow cytometry method is the most accurate method for separating X- and Y-chromosomes bearing sperm. However, the availability of this tool is very limited because the price is very expensive. Therefore, the sexing method with an albumin column using Bovine Serum Albumin (BSA) is an alternative that can be used.

Sexing spermatozoa with BSA is based on differences in motility and size. X-chromosome bearing sperm will remain in a medium that has a low concentration because its motility is smaller but the head size is larger. BSA media is made by forming fractions at various different concentrations, so that it meets the principles of this method and is suitable as an alternative material as a medium for separating X and Y sperm. Several factors influence the success of the sexing method with an albumin column, one of which is the incubation time. The incubation process is known to increase the production of free radicals which are very dangerous for sperm cells. Sperm plasma membranes contain many polyunsaturated fatty acids (PUFAs) which are very susceptible to reacting with free radicals. Spermatozoa contain a high concentration of PUFAs, which make them vulnerable to lipid peroxidation as lipid structures of the cell membranes are one of the primary targets for reactive oxygen species (ROS) [1]. PUFAs is the main component of sperm membranes and

maintaining the physiological conditions of sperm for survival. The testes and sperm have a characteristic lipid composition that is highly enriched in PUFAs [2-4]. Many researchers have reported that the addition of appropriate levels of PUFAs to semen extender could improve sperm antioxidant capacity and DNA integrity [5] and reduce oxidative stress levels [6]. PUFAs could improve sperm plasma membrane fluidity by trapping free radicals [7].

Several researchers have reported the effect of incubation time, including a positive correlation between the incubation time and sperm DNA damage, whereas prolonged incubation is associated with higher rates of sperm DNA fragmentation [8]. Also, the prolonged sperm incubation leads to a higher chromatin condensation and to a significantly increased number of DNA strand double breaks, but no influence on fertilization rates [9]. Moreover, the incubation time significantly affected the longevity of the sexed sperm of Pasundan bull, but not on plasma membrane integrity, abnormality, and DNA integrity [10].

Long incubation time cause the spermatozoa run out of energy to move and reduce the motility of the spermatozoa. However, if the incubation time is too long, the X and Y spermatozoa will not be completely separated and there will still be many spermatozoa carrying the Y chromosome left in the top fraction of the BSA solution. It has been reported that the incubation time of 45 minutes in the glutathione-based sperm sexing process is the optimum incubation time to maintain motility and intact plasma membrane (IPM) in chilled sexing semen of local sheep [11]. The length of incubation time also has a significant effect on reducing sperm motility in Boer goats [12]. It was also reported that incubation time significantly ($p<0.05$) effect on motility, IPM and intact acrosome cap (IAC), incubation time for 45 minutes obtained the longest longevity both at upper (7.5 days) and bottom (7.0 days) fraction, and the quality of sexed sperm of local ram fulfills the quality for artificial insemination and the incubation time for 45-75 minutes can be used to obtain the qualified sexed sperm for artificial insemination with chilled semen [13]. This study aims to determine the effect of incubation time on the quality of sperm resulting from post-thawing sexing in sheep sperm.

## MATERIAL AND METHODS

### Materials and Sexing Process

This study used three-year-old sheep ejaculate. The sheep were kept in individual pen and fed with mini elephant grass (*Pennisetum purpureum cv mott)* and tofu paste. Ejaculate was obtained through collection using an artificial vagina. Accommodation was carried out six times as repetition. Media used in the process of sperm sexing is BSA with column concentrations of 5% in the upper and 10% in lower fraction.

The ejaculate was diluted and placed on the surface of the BSA column and incubated at 37ºC. After the incubation process, sperm were collected from each 5% and 10% column BSA, and stored in a tube for later centrifuged Brackett-Oliphant medium for 10 minutes at 1800 rpm. Cell pellets sperm was added with Tris-egg yolk diluent 1 ml and stored at 5 ºC for 3 hours and then undergo the freezing process. Observations parameters were made after post thawing.

### Treatments and Parameters

This research used three incubation time: T1 (45 minutes), T2 (60 minutes), and T3 (75 minutes). Each treatment was repeated for six times. The parameters consist of motility, abnormality, intact acrosome cap, and recovery rate.

**Motility** calculations were carried out using a Neubaueur counting chamber. The motility value is obtained from the reduction of the total sperm concentration value by the dead sperm concentration, then divided by the total sperm concentration, and then multiplied by 100 percent.

**Sperm abnormality** is known through the differential coloring method using eosin dyes. The observed abnormalities are secondary abnormalities which include a severed tail, head without a tail, folded middle part. Observations were carried out under a microscope at 10x40 magnification. Abnormality of sperm was calculated from a minimum of 200 sperm.

**Intact Acrosome Cap** was evaluated by looking at the head of the spermatozoa which shows black color at the tip of the spermatozoa head, this shows that the acrosomal hood of the observed spermatozoa is still intact. The number of spermatozoa observed was 200 spermatozoa cells using a microscope with a magnification of 10x40.

**Recovery** rate is calculated by comparing post-thawing sperm motility with fresh motility multiplied by one hundred percent

### Research Design and Data Analysis

This research uses an experimental method with an experimental design, the completely randomized design (CRD). The experiment was carried out with three treatments where each treatment was repeated six times to obtain 18 experimental units. Data were analyzed using analysis of variance and Duncan's multiple follow-up test.

Table 1. The effect of incubation time on the quality of sexed sperm from upper fraction

| Parameters | T1 | T2 | T3 |
|---|---|---|---|
| | ……..…… % …… | | |
| Motility | 36.70 ± 2.30[a] | 34.10 ± 2.43[a] | 30.86 ± 2.31[b] |
| Intact Acrosome Cap | 31.50 ± 0.63[a] | 28.92 ± 0.58[b] | 26.92 ± 0.58[c] |
| Abnormality | 7.38 ± 1.70[a] | 7.75 ± 1.36[a] | 7.51 ±1.35[a] |
| Recovery Rate | 43.23 ± 2.67[a] | 40.12 ± 2.95[a] | 36.39 ± 2.55[b] |

Table 2. The effect of incubation time on the quality of sexed sperm from bottom fraction

| Parameters | T1 | T2 | T3 |
|---|---|---|---|
| | ……..…… % …… | | |
| Motility | 33.22 ± 3.20[a] | 29.94 ± 4.08[a] | 25.30 ± 2.82[b] |
| Intact Acrosome Cap | 30.75 ± 0.69[a] | 27.92 ± 0.38[b] | 25.83 ± 0.52[c] |
| Abnormality | 7.47 ± 1.13[a] | 7.7 ± 0.83[a] | 7.60 ± 1.54[a] |
| Recovery Rate | 39.13 ± 3.74[a] | 35.23 ± 4.84[a] | 28.83 ± 3.14[b] |

**RESULTS**

The effect of incubation time on the quality of sexed sperm from upper fraction is shown in Table 1, and from the bottom fraction is shown in Table 2.

Average sperm motility in the upper fraction ranges from 30.86 - 36.70%. The results of analysis of variance showed that the incubation time had a significant effect on motility in both top and bottom fractions, where Duncan's further test results showed that the incubation time of 45 minutes produced higher motility than the incubation time of 60 and 75 minutes in upper fractions, however between the incubation times of 60 and 75 minutes there was no significant difference.

The results of this study showed that the average IAC of sperm in the upper fraction ranged from 26.92 - 31.50%. The results of the analysis of variance showed that the length of incubation had an effect on the IAC in the upper fractions, where the results of Duncan's further tests in the upper fractions showed that the IAC resulting from the incubation time of 45 minutes was significantly higher than 60 and 75 minutes, and the IAC resulting from the long incubation period. incubation of 60 minutes was significantly higher than 75 minutes.

The average sperm abnormality in the upper fraction ranged from 7.38 - 7.75%. The results of analysis of variance showed that incubation time had no effect on sperm abnormalities resulting from sperm sexing. The average sperm recovery rate in the upper fraction ranged from 36.39 – 43.23%. The results of analysis of variance showed that incubation time had a significant effect on the recovery rate. Duncan's analysis results showed that incubation times of 45 and 60 minutes produced the best recovery rate compared to 75 minutes.

The results of this study showed that average sperm motility in the bottom fraction it is lower than the upper fraction, ranges from 25.30 - 33.22%. The results of analysis of variance showed that the incubation time had a significant effect on motility in bottom fractions, where Duncan's further test results showed that the incubation time of 45 minutes actually produced higher motility than the incubation time of 60 and 75 minutes in bottom fractions, however between the incubation times of 60 and 75 minutes there was no significant difference.

The average IAC of sperm in the bottom fraction it ranged from 25.83 - 30.75. The results of the analysis of variance showed that the incubation time had an effect on the IAC. where the results of Duncan's further tests in bottom fractions showed that the IAC resulting from the incubation time of 45 minutes was significantly higher than 60 and 75 minutes, and the IAC resulting from the long incubation period. incubation of 60 minutes was significantly higher than 75 minutes.

Same with upper fraction, the average of sperm abnormality in the bottom fraction it ranged from 7.47 - 7.60%. The results of analysis of variance showed that incubation time had no effect on sperm abnormalities. The average spermatozoa recovery rate in the bottom fraction

it ranged from 28.83 – 39.13%. The results of analysis of variance showed that incubation time had a significant effect on the recovery rate. Duncan's analysis results showed that incubation times of 45 and 60 minutes produced the best recovery rate compared to 75 minutes.

## DISCUSSION

Result of this research showed that the motility obtained was lower than SNI, namely 40%. Some of the contributing factors include the incubation process which is the sperm sexing process itself, the washing, centrifugation and freezing processes. The process of separating sperm from the upper to the bottom fraction requires energy obtained from metabolism, and as a by-product in the form of lactic acid which causes the pH to decrease and ultimately reduces sperm motility. The results of this study are in line with those reported by [11] that an incubation time of 45 minutes resulted in a motility of chilled sexed semen of 69.7% in the upper fraction and 68.8% in the bottom fraction. Other researchers have also reported that the incubation time affects the motility of cow sperm after sexing, where the incubation time of 20 minutes in the upper and lower layers (68.5% and 60.27%) is higher than the incubation time of 35 minutes (66.25% and 60%) and 50 minutes (65% and 52.5%) [14]. Likewise, research on Ettawah goats reported that an incubation time of 45 minutes resulted in higher motility (75.89%) in the upper fraction of sperm, while incubation times of 60 minutes and 75 minutes decreased, namely 70.57% and 68.26%. [15]. The results of ram chilled semen showed that both at upper and bottom fraction, incubation time had a significant ($p<0.05$) effect on motility [13].

Another factor that can reduce motility is when semen is washed with BO media using a centrifuge which results in damage to the sperm tails, so that their movement will decrease and the sperm may not move at all. The adverse impact of separating semen plasma using centrifugation techniques is the increased formation of ROS by sperm. The increase in ROS production by sperm after separation using centrifugation techniques is thought to be a complex process and can originate from various chemical processes, which occur in organelles or cells or originate from outside the cell. Various biological processes that can modulate the formation of ROS by sperm include modulating mechanical damage to the sperm membrane and the separation of seminal plasma from sperm. Seminal plasma is a medium for spermatozoa which consists of secretions from the testes, epididymis and accessory glands. The mixture consists of several factors such as organic and inorganic ingredients which are important in the sperm maturation mechanism under the control of hormones and enzymes. According to Aitken [16], ROS, and particularly their dismutase products $O2\bullet-$, $H2O2$, and hydroxyl radicals ($OH\bullet$), are capable of trespassing the cell membranes and disrupting the structure of various intracellular molecules such as proteins, lipids, and nucleic acids.

Motility is dependent on membrane integrity, because it is an important factor in sperm survival. The damage of membrane will be followed by decline of membrane integrity, motility, and sperm fertility. Sperm cell membrane phospholipids contain polyunsaturated fatty acids that are so high that they react easily with free radicals. The function of phospholipids is to maintain membrane integrity and as a protection against environmental conditions [17]. The long incubation time causes plasma membrane damage, this is because the longer the incubation time, resulting in the more polyunsaturated fatty acids present in the plasma membrane bind to free radicals, and lipid peroxidation occurs.

On the other hand, in this research the freezing process of course also reduces sperm motility. The freezing process causes sperm to experience cold shock due to very drastic changes in media temperature, as well as during the thawing process where the semen which was initially in a dormant state at a temperature of -196oC needs to adapt to the water temperature of 38oC in the vessel. The part of the sperm body that has the highest risk of damage is the tail, as stated by Januskauskas and Zillinskas [18] who said that decreased sperm motility due to freezing is believed to be related to mitochondrial damage. Sperm motility depends on mitochondrial function. Adenosine Tri Phosphate (ATP) is produced by oxidative phosphorylation in the mitochondrial membrane and transferred to microtubules for contraction of the fibrils in the principal piece and end piece of the spermatozoa tail which is then used for spermatozoa movement.

In the process of semen freezing, contact occurs between the semen and oxygen. Oxygen is an essential element, but excess oxygen causes peroxidative damage. Lipid peroxidation in sperm can increase abnormalities in the middle-broken tail or midpiece of spermatozoa, thereby allowing an increase in abnormalities. According to Olmo et al. [19] that incubation times that are too long trigger an increase in the production of free radicals. Excessive free radicals cause sperm to experience lipid peroxidation which can damage the integrity of the plasma membrane. Damaged plasma membranes can disrupt the metabolic process of sperm in producing energy for their movement (motility). Ram sperm cell membrane phospholipids are known to contain

a lot of PUFA which are very susceptible to free radical attack. According to Else and Kraffe [20], docosahexaenoic acid is one of the PUFA components that most actively reacts with free radicals and even has the highest level of formation of lipid peroxidation and aldehyde compounds compared to other components.

The factor that causes the motility of the upper fraction of spermatozoa to be greater than the motility of the lower fraction is because during the separation process, the sperm in the lower fraction require more energy to move further down towards the BSA solution which has a more concentrated concentration, so it is possible that the motility of the sperm will be very low might happen. Y-sperm, which are smaller in shape and size, and contain less DNA, have higher motility than X spermatozoa. Separation of sperm is based on differences in sperm head area, head length and DNA content as well as sperm movement, where Y-sperm have a smaller head area and lighter than X-sperm, the head length of Y- sper- is shorter with lower DNA content and faster movement than X-sperm.

The results of ram chilled semen were reported that both at upper and bottom fraction reported that incubation time had a significant ($p<0.05$) effect on intact acrosome cap. At upper fraction incubation time for 75 minutes obtained the highest IAC (68.85%), however at bottom fraction the highest IAC (72.05%) were obtained at 45 minutes incubation time [21].

Result of this research also showed that incubation time not significantly effect on the abnormality because during the incubation process, the upper fraction of spermatozoa does not experience much collision or friction between other sperm, because the principle of this incubation is to allow sperm to penetrate fractions of different concentrations, with their own motile power, so that the risk of damage to the tail or head of the sperm is not too high. big. This is what causes the incubation time to have no real effect on the percentage of sperm abnormalities. The results of this study are in line with the report by Rasad et al. [10] that stated incubation time has no effect on abnormalities.

Garner and Hafez [22] reported that the abnormality value for ram sperm was 5-20%. The number of sperm abnormalities reaching under 20%will not affect fertility. The form of sperm abnormalities observed does not indicate a primary abnormality, but is seen as an assessment of secondary abnormalities such as folded tails, broken heads and tails. The results of this study, although there was an increase in abnormalities compared to fresh semen, the abnormalities produced by sexed semen were still within the normal range. This increase is as a result of the freezing process.

The recovery rate value for the upper fraction with an incubation time of 45 minutes can be said to be close to the standard recovery rate value for bovine sperm based on SNI 4869-1 [23], namely 50%. Cell damage due to freezing can also occur due to dehydration, increased electrolyte concentrations, and the formation of intracellular ice crystals which can affect cell wall permeability and ultimately spermatozoa lose their motility [24]. Recovery rate functions to assess the ability of sperm to recover after going through the freezing process and shows the efficiency of the freezing process carried out. The higher the recovery rate value, the better the freezing process is carried out, which is related to the high level of sperm plasma membrane integrity which supports the metabolic process to produce good sperm movement.

The results of this study indicate that the incubation time of 45 minutes is the optimal incubation time for several sperm quality parameters such as motility, intact acrosome cap and recovery rate. Several previous studies support the results of this study, namely that an incubation time of 45 minutes produces longer viability than incubation times of 60 and 75 minutes [10, 20].

## CONCLUSION

Base on the result of the research it is concluded that incubation time significantly affected on the quality of post thawed ram sexed sperm, and 45 minutes is the optimal incubation time to provide the best quality.

## CONFLICT OF INTEREST

The authors declare no conflict of interest with any financial organization regarding the material discussed in the manuscript. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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