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The Quality of Frozen Semen with Different Thawing Duration and Temperature on Simmental Bull

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ABSTRACT: This study aimed to determine the accuracy of various thawing duration and temperatures to the quality of Simmental bull frozen semen. The experimental design used was a randomized group design with five treatments and ten replications, then analyzed using Chi-Square to compare the expected percentage of motility is 40%, and total motile spermatozoa are 10 million/straw value of SNI. A further test is carried out with Duncan's Multiple Range Test (DMRT) if there is a difference. The thawing treatments were 30 seconds at 37˚C water temperatures (T0) according to SNI and at 15 seconds (T1), 30 seconds (T2), 45 seconds (T3), and 60 seconds (T4) with 28˚C tap water temperatures. Variables observed involve motility, viability, abnormalities, concentration, and total motile spermatozoa. The data were analyzed using Analysis of Variance (ANOVA) with a significant difference in variance (P<0,05). The results showed that the different duration and temperatures of thawing influence motility (P<0,05) and the total motile spermatozoa, while the viability, abnormalities, and concentration had no influence (P>0,05). The motility and the total motile spermatozoa reach the highest results at the tap water temperatures T3 (P<0,05), while the lowest results were in T1 (P<0,05). It can be concluded that the different duration and temperatures of thawing influence motility and total motile spermatozoa. The best quality of Simmental bull frozen semen can be obtained with thawing using tap water at 28℃ temperatures for 45 seconds.

Keywords: Simmental bulls; Thawing; Tap water; Sperm quality

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INTRODUCTION

Improvements in reproduction and livestock production are carried out in natural mating procedures and artificial insemination (AI). The advantage of artificial insemination is improving genetics and the livestock population (Yeste, Rodriguez, and Bonet, 2016). Various factors influence the success of AI, including the thawing technique. Thawing treatment is carried out by inseminator using various temperatures and duration. According to National Standardization Agency of Indonesia (2021), thawing must be performed at 37℃-38℃ in 29 seconds, while the minimum motility of post thawing spermatozoa is 40%. However, many inseminators find it challenging to provide warm water, so they use tap water with temperatures between 25℃-30℃. According to Sari (2019), tap water has a temperature of 28℃. The quality of spermatozoa after thawing is not well studied. Therefore, it is necessary to research the thawing technique with a suitable duration and temperature by using tap water to get quality spermatozoa after thawing with SNI. This study aims to determine the effect of the thawing technique on the quality of post-thawing spermatozoa.

MATERIALS AND METHODS

The semen used were 50 frozen straw semen of Simmental bull produced by Singosari National AI Center Malang with batch code BBIB SINGOSARI SNI SIM SIGRA 61682 TT 0618.

The method was an experimental laboratory with the following treatments: thawing with warm water at 37℃ for 30 seconds (T0), thawing using cold water at 28℃ for 15 seconds (T1), thawing using cold water at 28℃ for 30 seconds (T2), thawing using cold water at 28℃ for 45 seconds (T3), and thawing using cold water at 28℃ for 60 seconds (T4).

Motility of Spermatozoa

To determine the value of motility is done by dripping one drop of semen on the object glass. After that, it was observed using a microscope with 400x magnification. Motility is calculated in 5 fields of view (Sumarandi, et al., 2019). Determining the spermatozoa percentage is done by assessing the number of live and progressive moving spermatozoa with a percentage of 0-100% (Ratnawati, Isnaini, and Susilawati, 2019).

Viability of Spermatozoa

Viability is obtained by dripping one drop of semen on the glass object and then mixing it with one drop of eosin-nigrosin. After that, it will be smeared using a glass object with 45 degrees deviation (Prastika, et al., 2018). Then it was observed by using a microscope with 400x magnification. The viability of spermatozoa was calculated with the formula:

$$\text{Viability} = \frac{\text{total live spermatozoa}}{\text{total spermatozoa counted}} \times 100\%$$

(Blegur, Nalley, and Hine, 2020).

Abnormality of Spermatozoa

The percentage of abnormality was obtained by dropping one drop of semen at the object-glass, and then it was dripped with eosin-nigrosin dye and smeared in 45 degrees. Then, it was observed using a microscope with 400x magnification and then calculated using the formula:

$$\text{Abnormality} = \frac{\text{total abnormal spermatozoa}}{\text{total spermatozoa counted}} \times 100\%$$

(Adnyani, Sumarandi, and Sarini, 2018).

Concentration of Spermatozoa

The concentration of spermatozoa can be determined by taking 10µl of semen using a micropipette. Then, add 990µl NaCl 3% homogenized for 2-3 minutes. After that, the cover glass will be placed on top of the Neubauer chamber, then dripped one drop on each side of the top and the bottom side. Then, spermatozoa were counted from 5 boxes on the upper right and left corners, the lower right and left, and the middle. To calculate the number of spermatozoa using the formula:

$$\text{Concentration} = \text{N} \times 5 \times \text{diluent factor} \times 10.000$$

Description:
N = mean number of spermatozoa in chamber
5 = 5 box count (correction factor)
FP = diluent factor (1:100)
10,000 = Neubauer chamber depth of 0.0001 ml/neubauer chamber (correction factor)
(Mahendra, Samsudewa, and Ondho, 2018).

Total Motile Spermatozoa

The total motile of spermatozoa is calculated from the multiply spermatozoa concentration with spermatozoa motility (Adhyatma, Isnaini, and Nuryadi, 2013). The total motile of spermatozoa was calculated after the percentage data of individual motility and spermatozoa concentration (million/straw) was obtained, then it can be calculated using the following formulation:

$$\text{TMS} = \text{Concentration of spermatozoa} \times \text{Individual Motility of Spermatozoa}$$

(Susilawati, 2013).

Statistic Analysis

The experimental design used was a randomized block design with five treatments and ten replications as a block. If there is a difference, further test is carried out with Duncan's Multiple Range Test (DMRT). Then, analyzed using Chi-Square as a comparison with the expected value of SNI. The first factor is thawing temperature, and the second factor is thawing duration. Observed variables include motility, viability, abnormality, concentration, and total motile spermatozoa.

RESULT AND DISCUSSION

1. Individual Motility of Spermatozoa

Table 1 shows the result of microscopic observations: the quality of post-thawing frozen semen Simmental bull spermatozoa with T0, T1, T2, T3, and T4 treatments.

Tabel 1. The average results of the post thawing frozen semen quality test

Treatment	Individual Motility (%)	Viability (%)	Abnormality (%)	Concentration (Million/Straw)	Total Motile Spermatozoa (Million/Straw)
T0	55.2 ± 3.9^{e}	69.0 ± 7.9	8.5 ± 3.1	24.5 ± 5.0	13.5 ± 2.6^{c}
T1	33.4 ± 3.0^{a}	69.4 ± 11.8	4.7 ± 2.3	20.8 ± 4.7	7.0 ± 1.8^{a}
T2	41.5 ± 2.9^{b}	63.0 ± 2.5	5.8 ± 1.3	28.3 ± 6.0	11.7 ± 2.7^{bc}
T3	50.8 ± 4.0^{d}	65.8 ± 6.1	7.5 ± 3.3	26.3 ± 5.3	13.4 ± 3.1^{c}
T4	46.0 ± 5.3^{c}	65.5 ± 8.6	6.9 ± 3.4	27.9 ± 9.9	12.8 ± 4.8^{c}

Description: Different notation shows a significant difference ($P<0.05$)

Based on Table 1. the interaction between 2 factors had a significant effect (P<0.01) on the individual motility and total motile spermatozoa. In contrast, the interaction of the two factors on viability, abnormality, and concentration had no significant effect (P>0.05). According to SNI 4869-1:2021, the frozen semen must have a 40% minimum of spermatozoa post thawing. Based on the result of the study in table 1, the best motility average is 55,2%±3,9% at the control treatment (T0). In contrast, the best motility average from thawing treatment using tap water is 50,8%±4,0% (T3).

The result of statistical analysis showed that the frozen semen treatment had a significant difference (P<0.05) in individual motility. Nalley, Pratama, and Arifiantini (2016) mention that the motility parameter was observed from the movement speed of individual spermatozoa moving forward, which was expressed in a score. The minimum score for good semen quality is 2. Due to the influence of the treatment on the motility of individual spermatozoa, Duncan's multiple-distance further test was carried out. The results of Duncan's test showed that the control treatment (T0) had a significant effect on the percentage of individual motility of post-thawing frozen semen of Simmental bull. Meanwhile, all replicates had the same effect on the individual motility percentage of post-thawing frozen semen of Simmental bull. Then the results of statistical analysis using Chi-Square showed that at T0 and T3, the percentage of individual motility was higher than the expected value. While at T1, T2, and T4, the percentage of individual motility was close to even lower than the expected value.

The observation results showed that the motility of individual spermatozoa decreased when using different water and temperatures and duration from SNI. The wrong water temperatures and duration can cause the optimal miss point of spermatozoa to move progressively. The motility of spermatozoa post thawing can decrease if there is an error in determining the thawing method's standard, duration, and temperature (Susilawati, 2013). Spermatozoa began to live again after the treatments. However, the longer duration, the higher the metabolism activity, which needs more energy. The lactic acid production will increase inside spermatozoa, which is toxic, so it will reduce the movement momentum until death occurs. Spermatozoa activity requires ATP.

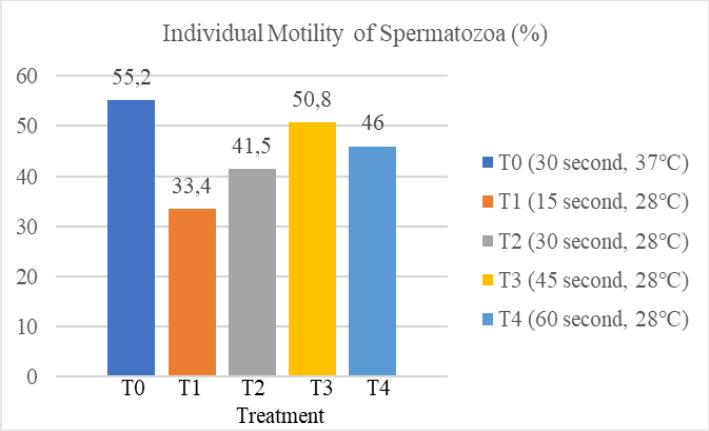

Figure 1. The average of spermatozoa individual motility

The use of ATP is controlled by endogenous levels and Adenosine Monophosphate (cAMP), so it influences the motility of spermatozoa (Susilawati, 2011). ATP is a source of energy for cells that are produced through biochemistry in mitochondria. According to Salim, Susilawati, and Wahyuningsih (2012), the increase of free radicals such as Reactive Oxygen Species (ROS) can cause damage

to the mitochondrial membrane structure that can cause apoptosis or physiological cell death due to changes in morphology and cell biochemistry. If the function of the cell membrane has a problem, the ability of the cell to synthesize ATP will decrease, which then affects the optimal point of moving spermatozoa.

Picture 1 showed that the lower the thawing temperature, the longer duration required than the standard duration from SNI at its optimal point. However, the results showed that the controlled treatments and the treatment that has been adjusted to field conditions were still better than the treatments based on SNI standards, with a 4,4% decrease from the best treatments.

2. Viability of Spermatozoa

The result of statistical analysis showed that the two factors in the thawing treatments had no significant effect (P>0,05) on spermatozoa viability, so no further DMRT test was carried out. Spermatozoa viability above 70% is excellent because the viability percentage must be higher than the motility percentage (Nugroho, Susilawati, and Wahjuningsih, 2014). According to Garner and Hafez (2000), the viability of frozen spermatozoa after the thawing process is 60% minimum up to 75%. The study showed that the highest percentage of viability is 69,4% ± 11,8% (T1), while the lowest percentage is 63% ± 2,1% (T2). The lower total live spermatozoa indicate that the quality of semen is getting worse.

The viability observations in picture 2 show that T0 is different from T2, even though it uses the same time duration. The percentage of viability of T0 (P>0.05) was 69.0% ± 7.9%, but T2 (P>0.05) only reached 63.0% ± 2.5%, much lower with a difference of 6%. While the treatment T0 (P> 0.05) with T3 (P> 0.05) gave different results, with a difference of 3.2%. Observations showed that the control treatment (T0) was lower than T1, but T4 was lower than the control (T0). This shows that determining the viability of many factors can influence, so the results shown can be different. The time and thawing temperature factor in all treatments did not significantly affect the viability percentage to below. Five treatments showed the appropriate viability, which was >60%, it could still be used for AI. Thawing time and temperature do not cause extreme changes.

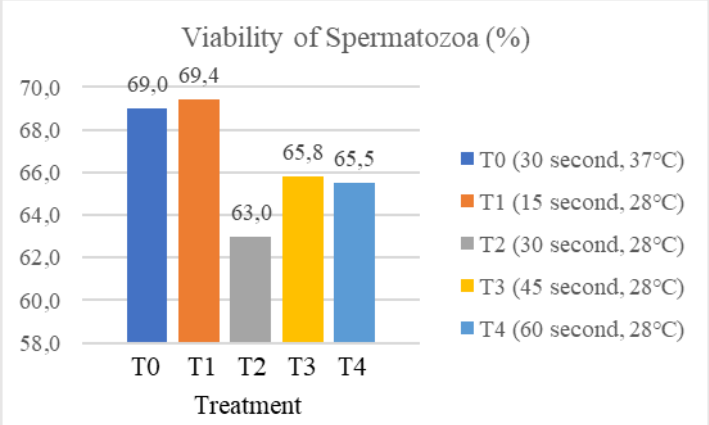

Figure 2. The average of spermatozoa viability

The pressure of preparation when make a smear can affect the viability of spermatozoa. The too intense pressure can damage the spermatozoa membrane, indicating it is damaged. In addition, the short thawing duration causes the metabolism of spermatozoa cells that are not optimal, and lactic acid has not fully increased. Hence, the viability of cells is still high. Increased activity can cause fatty acids to be high due to chemical interactions, namely lipid peroxidation. Protein lipids and carbohydrates are constituents of cell membranes, so if there is an inappropriate

extracellular influence, it can cause the death of spermatozoa. Spermatozoa discoloration is influenced by cell damage due to handling during thawing and genetic damage. Spermatozoa with redheads are classified as dead spermatozoa, while spermatozoa with transparent or colorless heads are classified as live spermatozoa (Fitrik and Supartini, 2012). Spermatozoa that absorb color indicate that the cell has died because the acrosome cells are not working properly to reject other substances that enter the sperm cell (Rohmah, Santoso, and Zayadi, 2020). Spermatozoa membranes that are still goodwill prevent foreign substances from entering the cell.

3. Abnormality of Spermatozoa

The results of the analysis showed that the two factors in the thawing treatment had no significant effect (P>0.05) on spermatozoa abnormalities, so further DMRT testing was not carried out. According to Das, et al., (2020), frozen semen has a maximum abnormality proportion of 20%. Based on the study results in table 1, the best percentage of abnormalities in thawing treatment using tap water is 4.8% ± 2.2% (T1), while the highest proportion is 7.3% ± 3.2% (T3). The higher the percentage, the lower the semen quality. High abnormality indicates many levels of damage or disproportionate proportions of spermatozoa.

The tail is present in spermatozoa due to osmotic pressure at the time of dilution of semen and cold shock during the cooling treatment process in semen (Sugiarto, Susilawati, and Wahyuningsih, 2014). Severed tails in spermatozoa were caused by two assumptions, the first because of an error when make a smear that was too harsh or pressing. At the same time, the second assumption is a detached spermatozoa acrosome (Sades, Isnaini, and Wahjuningsih, 2016).

It can be seen that the factor of thawing time and the temperature does not have a significant effect on spermatozoa abnormalities. Many other factors affect the viability of spermatozoa post thawing. This is confirmed again by picture 3, which shows fluctuating or inconsistent results. The results obtained are still in the good category because the average percentage of abnormalities is <20%, so it is feasible to use artificial insemination.

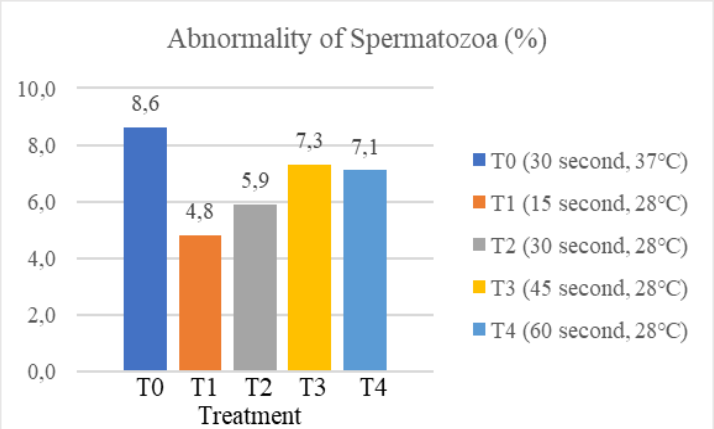

Figure 3. The average of spermatozoa abnormality

4. Concentration of Spermatozoa

The statistical analysis results showed that the length of time and temperature had no significant effect (P>0.05) on the concentration of spermatozoa, so no further DMRT test was carried out. According to the National Standards Agency in SNI 4869-1:2021, the minimum standard of spermatozoa concentration is 25 million per dose. The Chi-Square test results showed that the concentration of spermatozoa per straw in T2, T3, and T4 was above the expected value of 25 million/straw, while T0 and T1 were below the expected value of 25 million/straw.

Based on the research, results in table 1 and picture 4 showed the highest

concentration of frozen semen (T2), while the lowest concentration of spermatozoa is 20,8±4,7 million/straw. T0 and T1 have an average below the standard, while T2, T3, T4 have an average above the standard. In the observation results, the control, which should have a standard according to SNI, was after the quality test gave different results. This indicates that the thawing time and temperature factors did not affect the concentration of spermatozoa. The concentration of spermatozoa can be affected by the volume of semen. The higher the volume of semen it will be directly proportional to the number of spermatozoa (Nyuwita, Susilawati, and Isnaini, 2015). The concentration of spermatozoa in the straws can be different, which is forgotten because during the dilution process, the inclusion of semen in the straws is not evenly distributed. However, in one production, the possibility that differences in the concentration of spermatozoa in each straw could occur.

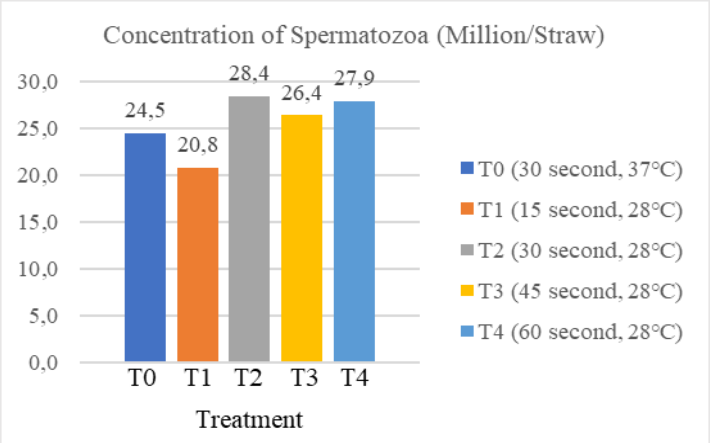

Figure 4. The average of spermatozoa concentration

Spermatozoa concentration is one of the factors supporting fertilization. Although there is only one spermatozoa cell in the fertilization process that can penetrate the zona pellucida and the ovum cell membrane, a standard number of spermatozoa is still required. This is so that the possibility of surviving spermatozoa while traveling to the fallopian tube for fertilization can be greater. When traveling to the fallopian tube, Spermatozoa require a large amount of energy so that the metabolism of spermatozoa cells will increase. Increased metabolism will produce high lactic acid, killing the spermatozoa cells themselves. Many are trapped in the cervical crypt mucosal folds (Yekti, et al., 2017).

5. Total Motile Spermatozoa

The analysis results showed that the frozen semen treatment for the Simmental bull had a significant difference (P<0.05) in the total motile spermatozoa. Duncan's test results show that T0, T3, and T4 significantly affected the total percentage of motile spermatozoa frozen semen of Simmental bull post thawing. However, the effect did not show any difference with T2. Meanwhile, all replicates had the same effect on the percentage of total motile spermatozoa in frozen semen of Simmental bull post thawing. Total Sperm Motility or TSM calculates the number of motile spermatozoa by multiplying the motility of individual spermatozoa by the total number of spermatozoa (Adhyatma, Isnaini, and Nuryadi, 2013).

In the Chi-Square test, the expected value of the total motile spermatozoa is about 10 million/straw from the SNI provisions, at T0, T3, and T4 the total spermatozoa were higher than the expected value. While in T1 and T2, the results are lower and closer to the expected value. Based on the research in table 1, shows that the highest number of motile spermatozoa

in frozen semen of Simmental bull is 13.5±2.6 million/straw (T0), while the lowest total motile spermatozoa are 7.0±1.8 million/straw (T1).

The results of observations from picture 5 showed that the total frozen semen motile spermatozoa from T1 were not according with SNI because they were below 10 million/straw, while the total frozen motile spermatozoa from T0, T2, T3, T4 were according with SNI, which was above 10 million/straw. The number of motile spermatozoa was obtained by multiplying the motility of individual spermatozoa by the total number of spermatozoa (Adhyatma, Isnaini, and Nuryadi, 2013). Motility is strongly influenced by the length of time and temperature of thawing so that it also affects the number of motile spermatozoa in frozen semen after thawing.

The use of tap water with 45 seconds gave better results than the time variations in other treatments. This indicates that the number of spermatozoa moving forward is still above the minimum requirement for semen to be inseminated. This statement is supported by the opinion of Hanifi, Ihsan, and Susilawati (2016) who states that the calculation of the total motile spermatozoa is used for semen selection in artificial insemination. The higher the total motile spermatozoa, the better the semen quality. The number of motile spermatozoa can determine the chances of fertilization (Sugiarto et al, 2014).

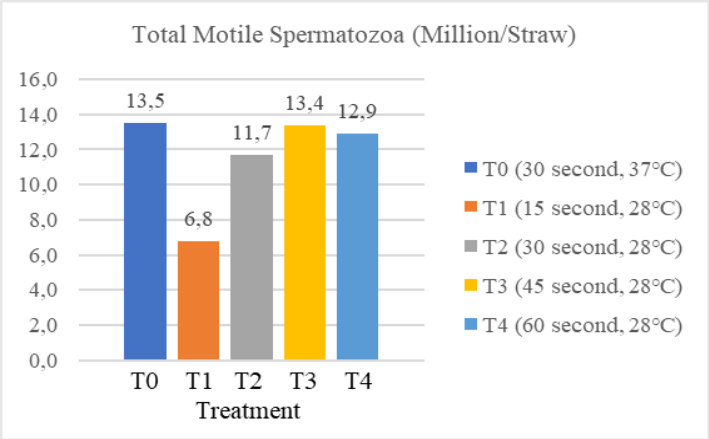

Figure 5. The average of total motile spermatozoa

High motility and high concentration will be directly proportional to the total motile spermatozoa. In T1 the results of low motility only reached 33.4%±3.0%, and the concentration reached 20.8±4.7 million/ml, so the total motile spermatozoa were also low, which only reached 7.0±1.8 million/ml straw.

In contrast to T3 with motility reaching 50.8%±4.0% and spermatozoa concentration reaching 26.3±5.3 million/ml, high total motile spermatozoa reaching 13.4±3.1 million/straw. Even though the total motile spermatozoa in T3 was better than T1, T2, and T4, however the best quality was still using the appropriate temperature and length time according to SNI (T0).

CONCLUSIONS

Thawing duration and temperature affect the motility and total motile spermatozoa but do not affect spermatozoa's viability, abnormality, and concentration. The quality of spermatozoa using tap water temperature of 28°C for 45 seconds showed the best results, approaching the SNI standard with average individual motility of 50.8%, viability 65.8%, abnormalities 7.3%, concentration 26.4 million/straw, and total motile spermatozoa 13.4 million/straw.

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