

# Effect of Incubation Time During Sperm Sexing Process on Sperm Quality of Pasundan Bull

Rasad SD, Solihati N, Winangun K, Yusrina A, Avicenna F

Animal Husbandry Faculty, Padjadjaran University, Jl. Raya Bandung Sumedang KM 21, Jatinangor, Sumedang  
E-mail: s.d.rasad@unpad.ac.id

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## ABSTRAK

Rasad SD, Solihati N, Winangun K, Yusrina A, Avicenna F. 2020. Pengaruh waktu inkubasi selama proses *sexing* sperma pada sperma kualitas Pasundan Bull. JITV 25(3): 112-119. DOI: <http://dx.doi.org/10.14334/jitv/v25i3.2494>

Tujuan dari penelitian adalah untuk menguji pengaruh waktu inkubasi terhadap kualitas semen *sexing* Sapi Pasundan. Separasi sperma menggunakan Bovine Serum Albumin (BSA) dengan konsentrasi 5% dan 10%, dengan perlakuan waktu inkubasi P1 = 45 menit, P2 = 60 menit dan P3. 75 menit. Penelitian ini menggunakan metoda Rancangan Acak Lengkap (RAL) dengan tiga perlakuan dan 6 ulangan. Data dianalisis dengan Duncan *test*. Parameter yang diukur adalah daya tahan hidup, membran plasma utuh, abnormalitas dan integritas DNA sperma *sexing*. Hasil penelitian menunjukkan waktu inkubasi berpengaruh ( $P < 0,05$ ) terhadap daya tahan hidup, tetapi tidak pada MPU, abnormalitas, dan integritas DNA. Waktu inkubasi 45 menit menghasilkan daya tahan hidup terbaik pada fraksi atas (4,33 hari) dan fraksi bawah (4,17 hari). Abnormalitas sperma pada fraksi atas antara 4,00%-4,50% dan fraksi bawah antara 4,10%-4,40%, sedang integritas DNA pada fraksi atas antara 98,16%-98,66% dan fraksi bawah antara 97,83%-98,58%. Dari hasil penelitian dapat disimpulkan bahwa waktu inkubasi berpengaruh terhadap daya tahan hidup sperma, tetapi tidak terhadap MPU, abnormalitas dan integritas DNA sperma Sapi Pasundan hasil *sexing*

**Kata Kunci :** Waktu Inkubasi, Sapi Pasundan, *Sexing* Sperma

## ABSTRACT

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The research was conducted to evaluate the effect of incubation time on viability, plasma membrane integrity, abnormality, and DNA integrity of sexed Pasundan's bulls sperm. The sperm sexing used 5% and 10% concentrations of Bovine Serum Albumin (BSA). A completely randomized design with three treatments and six replications was used in this study. The data were analyzed using variance analysis followed by Duncan's multiple distance test. Parameter evaluated were sperm longevity, plasma membrane integrity (PMI), abnormality, and DNA integrity of sexed Pasundan bulls sperm. Results showed that incubation time gave significant effect ( $P < 0.05$ ) on the longevity of sperm, but not on the PMI of Pasundan bulls sexed sperm. The incubation time of 45 minutes gave the highest value of longevity sperm on the upper layer (4.33 days) and the lower layer (4.17 days). Furthermore, the abnormality of sperm X in the upper layer was 4.00%-4.20% and the lower layer was 4.10%-4.40%. Meanwhile, the DNA integrity of an upper layer was 98.16%-98.66%, and the lower layer was 97.83%-98.58%. It is concluded that 45 minutes of incubation time significantly affected the longevity of sperm, but not plasma membrane integrity, abnormality, and DNA integrity of Pasundan bulls sexed sperm.

**Key Words:** Incubation Time, Pasundan Bull, Sexed Sperm

## INTRODUCTION

Artificial Insemination technology could be increased in value using a sexed semen program that produces expected sex of calf, and this sex predetermination is commercially crucial in the farm (Kusumawati et al. 2017). Sperm sexing technology in livestock is an effort to increase the chances of certain birth of certain sex offspring according to the purpose of livestock raising. The application of sperm sexing

technology could improve the efficiency of the Artificial Insemination (AI) program.

Sperm sexing is a method of separation between X chromosome bearing sperm (X-sperm) and Y chromosome bearing sperm (Y-sperm) to obtain a higher proportion of certain sperm to get a higher probability of birth of certain sex (Solihati et al. 2019). A direct method for sperm sexing in animals is based on the sorting of X and Y-bearing sperm before insemination. Sperm are separated into X and Y-

bearing populations based on the presence of X or Y chromosomes, respectively. The egg contains only X-chromosome then be fertilized by an X-sperm will produce female offspring, while if it is fertilized by a Y sperm, the offspring will be a male. Therefore, sexing spermatozoa could be used in conjunction with artificial insemination to produce expected offspring sex (Yadav et al. 2018). Even though sperm sexing contributes a great impact on breeding programs, it requires high cost and resulted in a low pregnancy rate especially when using the artificial insemination program (Carvalho et al. 2010). Separation of X and Y chromosomes could be done by the sedimentation method with Bovine Serum Albumin (BSA) solution. Carvalho et al. (2010) reported that the Y chromosome is smaller than the X chromosome. In previous studies, damage of spermatozoa membrane in the sexing process with albumin gradient could decrease the quality of spermatozoa, thus diluents for protecting the spermatozoa membrane to be in good quality are needed. The decrease in semen quality is due to physical and chemical effects on sperm cells during the separation process to obtain X or Y sperm.

The length of incubation is one factor influencing the sexed sperm quality. Incubation time is the time needed for the sperm to penetrate the BSA solution during the sexing process. Sperm containing an X chromosome is larger than those containing a Y chromosome. Different incubation times are estimated to affect the quality and longevity of X and Y sperm, but the information is still limited. Some researchers had performed sperm sexing with a 60-minute incubation period (Gunawan 2015; Hadi & Al-Tamimi 2013), and shorter incubation times for 20, 35 and 50 minutes (Sunarti, T Saili, et al. 2016), and 10, 20, 30 minutes (Situmorang et al. 2013), and 30, 60, 90, 120 minutes (Afriani et al. 2011).

One of the sperm quality results from the sexing process that needs to be considered is its viability, plasma membrane integrity, and abnormality because it is closely related to fertility. In addition to abnormalities, the DNA integrity of sperm also needs to be considered, because DNA is a genetic carrier for the offspring (Chowdhury et al. 2014). Based on the reasons above, this study was conducted to determine the effect of incubation time on sexed semen quality of Pasundan cattle.

## MATERIALS AND METHODS

### Experimental animals

This research was done in October - December 2018 at the Center of Beef Cattle Breeding Development and Artificial Insemination Institute, Cibeunghing, Ciampis. Semen was collected from seven five year Pasundan bulls, which was certificated from Indonesian Product

Certification Institute. All bulls were maintained in semi-intensive cages, located in Cibeunghing, Ciampis, which was feed daily in form of forages and concentrates, as usual feeding in that center.

### Semen collection and evaluation

BSA and BO solution was prepared one day before semen collection. Collecting semen was done twice a week and directly evaluated on macroscopic and microscopic quality.

Fresh semen was prepared and diluted using BO solution with a ratio of fresh semen and BO solution as 1:4. As much as 2 ml of 10%, and 5% BSA solutions respectively were slowly placed into tubes to make the BSA column. The diluted semen was put into the BSA column as much as 1 ml, so that the ratio of semen and BSA was 1: 4, respectively. The tubes were incubated into a water bath at 37°C for 45 minutes, 60 minutes, and 75 minutes. During incubation, spermatozoa would penetrate the solution based on the speed of movement and form 3 layers: upper layer containing immotile sperm, the middle layer containing X sperm, and the lower layer containing Y sperm. One ml of the top layer was considered immotile and removed. Four milliliters of each BSA solution were separated and transferred into centrifugation tubes, then labeled as Y for the 10% BSA solution and X for the 5% BSA solution. Five ml BO solution was added to each centrifugation tube. They were centrifuged at 1800 rpm for 10 minutes. The centrifugation process formed 2 layers: the top layer was a supernatant that will be removed and the bottom layer was a pellet which was the result of sperm sexing. The tubes containing pellets were added by BO solution slowly through the tube wall. Semen was diluted with TRIS-egg yolk, then stored at 5°C as liquid semen.

The treatment of this research was incubation time and carried out after the sperm sexing process for 45 minutes incubation time ( $T_1$ ), 60 minutes incubation time ( $T_2$ ) and 75 minutes incubation time ( $T_3$ ). There were 6 replications. The test is based on the number of bulls from which the sperm is taken. Dilution of semen used was TRIS-egg yolk.

### The longevity of sperm observation (day)

The longevity of sperm was observed automatically using Computerized-Assisted Sperm Analysis (CASA) (AndroVision, MiniTube) connected to the microscope. The data was seen from the survival of sperm after treatment in units of days.

### Plasma membrane integrity (PMI) (%)

Plasma membrane integrity was evaluated using a hypo-osmotic swelling test (HOS-Test) solution made

from 0.179 grams of 0.1 M NaCl dissolved into 100 ml of bidistilled water, semen samples were put into hypo-osmotic solution, then incubated for 30 minutes at 37°C. After incubation, sperm were evaluated using an Olympus CX-21 binocular microscope with a 40x10 magnification. Sperm that have an intact plasma membrane is characterized by a swollen, circular tail, due to exposure to hypotonic solutions. Damaged sperm is characterized by a straight tail due to the absence of an osmotic reaction.

#### Abnormality of sperm (%)

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.

#### DNA integrity (%)

This observation was seen by the Acridine Orange Test method and observed under a fluorescence microscope. Sperm with normal DNA shows greener fluorescence, while sperm with fragmented DNA will emit fluorescence that varies from yellow-green to orange. The percentage of sperm with whole chromatin was calculated by dividing the sperm count normal (green) with a total of 200 sperm counts observed.

#### Statistical analysis

The Completely Randomized Design (CRD) with three treatments and six replications was applied. Data were analyzed using variance analysis followed by Duncan's multiple distance test. The parameters of this study include viability, plasma membrane integrity, abnormality, and DNA integrity of the sexed sperm of Pasundan bull.

**Table 1.** The macroscopic and microscopic evaluation of fresh Pasundan bull semen

Parameter	Replication						Average
	1	2	3	4	5	6	
<b>Macroscopic:</b>							
Volume (ml)	8.00	5.50	5.00	6.00	5.00	5.50	5.831
Colour	Creamy white	-					
Consistency	moderate	moderate	Aqueous	moderate	moderate	moderate	-
pH	6.40	6.40	6.40	6.40	6.40	6.40	6.40
<b>Microscopic:</b>							
Mass activity	++	++	++	+++	++	++	-
Total Sperm-Concentration per ejaculate (million/ml)	1,503.00	1,358.00	1,148.00	1,600.00	1,210.00	1,312.00	1,355.17±171.85
Sperm Motility (%)	70.00	70.00	70.00	70.00	70.00	75.00	70.83±2.04
Sperm Abnormalities (%)	3.00	3.00	3.00	2.50	2.00	2.50	2.70±0.40
DNA- Integrity (%)	98.00	99.00	98.00	98.00	99.00	98.00	98.33±0.47
Membrane plasma integrity (IPM)(%)	65.00	62.00	65.00	69.14	66.52	73.17	66.81±3.89

## RESULTS AND DISCUSSION

### Fresh semen evaluation

The evaluation of the fresh semen samples was done immediately after semen collection. The result of the evaluation is presented in Table 1. Table 1 shows the average semen volume was 5.83 ml. This result is in line with Feradis (2010); Kedia et al. 2014 and Anwar et al. (2015) which stated that the volume of bulls semen ranges from 5-8 ml /ejaculation. The color of semen in this study was creamy white, this is in line with Tan et al. (2014) and Solihati et al. (2018) which stated that the color of bull semen is milk-white and creamy.

Normal semen obtained had a distinctive odor, and thick consistency, this is in line with the result of (Kedia et al. 2014). The pH of the obtained semen was 6. The pH of fresh semen ranges from 6.4-7.8 (Ax et al. 2000). The mass activity of Pasundan bulls semen was ++ which was good because it looked like a big wave, many, dark, thick, and actively powered according to (Baharun et al. 2017). The sperm motility was 70.83% moved progressively, the motility of the individual was still in the normal range and in line with (Ax et al. 2000) who stated that the sperm motility of bull was 70%. Sperm concentration was 1.355 million/ml. This is in line with (Feradis 2010) who found the concentration of fresh semen of bulls was 1.000–2.000 million sperm cells per ml.

The percentage of sperm motility of Pasundan bulls was 70.83%. This result is lower than (Baharun et al. 2017) which was  $82.41 \pm 2.97\%$ . The difference could be influenced by sperm maturity and the quality of plasma semen (Komariah et al. 2013) but these results were optimal according to Ax et al. (2000), the percentage of sperm motility must be more than 50%.

The percentage of spermatozoa abnormalities was 2.70%. This is smaller than the average abnormalities of Pasundan cattle of (Baharun et al. 2017) which was  $11.13 \pm 0.39\%$ . According to Sujoko et al. (2009) abnormalities in ram sperm ranged from 5.00% to 35.00%, the abnormality of Pasundan bulls sperm in this study was very low. Abnormality is one indicator determining sperm quality. The abnormal morphology of sperm cells will affect the fertilization process, the high abnormality of spermatozoa will reduce fertility (Sujoko et al. 2009).

The IPM obtained in this study was 62.00% to 73.17% with an average of  $66.81 \pm 3.89\%$ . This result was lower than that of Baharun et al. (2017), which was  $84.89 \pm 1.00\%$ . The percentage of IPM that have optimum quality and requirements for further processing is  $\geq 60.00\%$  (Rizal et al. 2013; Shukla et al. 2013). The damage of plasma membranes cause decreased cell membrane integrity so that control of the

transport system in sperm cells is disrupted which resulted in the decrease of metabolism, motility, and survival of spermatozoa (Purwoistri et al. 2013; Mishra et al. 2013).

### Effect of incubation time on the longevity of sperm

The longevity of sperm was evaluated by looking at their motility daily until the motility reaches a minimum percentage of 40.00%. Table 2 shows the incubation time of 45, 60, and 75 minutes result in a significant effect ( $P < 0.05$ ) on the longevity of sperm at the upper and lower layer. The 45 minutes of incubation time produced the longest longevity, while 75 minutes of incubation time produced the shortest longevity of sperm. This result was longer than that of Solihati et al. (2018) average longevity of sperm ranged between 13.875–19.625 hour. This result indicated that the longer the incubation time, the lower the longevity of sperm is.

The longevity of sperm is closely related to sperm motility because sperm longevity is obtained by observing sperm motility. The longevity of sperm at the upper layer was observed to a minimum of 40% motility. Decreasing the percentage of spermatozoa motility after sexing due to the long incubation time in the albumin layer, speed, and length of centrifugation which causes sperm losing a lot of energy thereby reducing the level of motility (Sudarma et al. 2014). The incubation process decreased the viability of the upper layer sperm from T1 to T3 because, during the incubation process, free radical formation occurs including hydrogen peroxide ( $H_2O_2$ ) which is toxic and causes the damage of the plasma membrane, so that sperm motility decreases (Storey 2008). Hydrogen peroxide ( $H_2O_2$ ) is needed for sperm motility and is useful in the capacitation process, so that sperm cells are produced naturally, but if the level is too high, it will cause lipid peroxidation. Hydrogen peroxide ( $H_2O_2$ ) will react with polyunsaturated fatty acids that are in the sperm plasma membrane, causing lipid peroxidation. Damage of the plasma membrane due to lipid peroxidation directly decreases the motility of sperm. Decreased sperm survival is also due to the length of storage, because the longer storage time, lactic acid formed due to the process of sperm cell metabolism increases, and causes a high pH of the medium. Rizal & Riyadhhi (2016) stated that sperm in anaerobic storage conditions (without oxygen) occurs in the process of cell metabolism which results, in the end, is lactic acid. The duration of storage causes a buildup of lactic acid in the media so that the pH of the media drops and results in the death of spermatozoa. Table 2 shows that Y sperm has a smaller and lighter head than X sperm, this is because Y sperm has a smaller amount of chromatin than X sperm, so it will have a direct impact

on sperm motility. Y sperm move faster than X sperm. Therefore Y sperm lose more energy quickly due to movement (Sunarti et al. 2016). The average motility of sperm in the lower layer is smaller than upper layer sperm, this is due to the lower layer sperm passing through two layers so that more energy is used, resulted in a decrease in motility, and viability. Sperm storage for a long time also caused motility to decrease, due to the presence of lactic acid resulting from cell metabolic processes, so that the pH of the medium is more acidic.

**Effect of incubation time on plasma membrane integrity (PMI) of sperm after sexing process**

The average PMI of sperm at the upper and lower layer after sexing using different times of incubation is presented in Table 3.

The sperm plasma membrane is the outer part that restricts between inside and external environments of sperm cells and rewarding in the process of metabolism of cells (Ode et al. 2010). Damage of the plasma membrane will affect the process of sperm metabolism so that it will affect the motility of sperm.

The results of the analysis of variance showed that the length of incubation did not significantly affect PMI in the upper layer.

Based on the observations, the percentage of plasma membrane integrity produced in this study after the sexing process did not differ from that of Sunarti et al. (2016). The average intact plasma membrane spermatozoa of Bali cattle after sexing using albumin of white egg and density gradients with each sexing time of 20 minutes, 35 minutes, and 50 minutes had no significant effect on PMI with an incubation time of 20 minutes, 35 minutes, and 50 minutes was  $81.63 \pm 28.93\%$ ,  $81.14 \pm 28.77\%$ , and  $80.57 \pm 28.91\%$ .

Sperm plasma membrane integrity is an important factor in sperm survival, because membrane damage will be followed by decreased of membrane integrity, motility, and spermatozoa fertility. Sperm cell membrane phospholipids are known to 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 (Diliyana et al. 2014). The long incubation time causes plasma membrane damage, this is because the longer the incubation time, the more polyunsaturated fatty acids present in the plasma membrane bind to free radicals, and lipid peroxidation occurred. So in this study, the incubation time treatment that produced the highest percentage of plasma membrane integrity was 45 minutes and the lowest was 75 minutes.

**Effect of incubation time on the abnormality of sperm after sexing process**

The effect of incubation time on the abnormality of sperm after sexing using BSA can be seen in Table 4. The result of the statistical test showed that incubation time was not significantly different on the abnormality of sperm after sexed in the upper and lower layer. This is may due to BSA content, diluents, incubation time and storage for 4 hours at 5°C does not affect the morphological condition of Pasundan bull sperm.

The content of BSA can protect the sperm. In line with Uysal & Bucak (2007) which states that the composition of BSA is to protect the integrity of the spermatozoa membrane from environmental conditions such as heat or oxidative conditions. Bovine serum albumin (BSA) are extracellular cryoprotectants as a function of cell plasma membrane protectors. Bovine

**Table 2.** Effect of incubation time during sperm sexing process on the longevity of sperm

Replication	Treatment					
	Upper layer			Lower layer		
	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>
.....days .....						
1	4.00	4.00	3.00	5.00	3.00	3.00
2	4.00	3.00	3.00	4.00	4.00	3.00
3	4.00	4.00	3.00	4.00	4.00	3.00
4	4.00	4.00	3.00	4.00	4.00	3.00
5	4.00	4.00	4.00	4.00	3.00	4.00
6	5.00	4.00	4.00	4.00	3.00	4.00
Total	25.00	23.00	20.00	25.00	21.00	20.00
Average	4.17±0.41 <sup>a</sup>	3.83±0.41 <sup>b</sup>	3.33±0.52 <sup>b</sup>	4.17±0.41 <sup>A</sup>	3.50±0.55 <sup>B</sup>	3.33±0.52 <sup>B</sup>

P1: 45 minutes of incubation time, P2: 60 minutes of incubation times, P3: 70 minutes of incubation time  
 Different superscripts within row show significant differences. a,b upper layer; A,B lower layer

**Table 3.** Effect of incubation time during sperm sexing process on intact plasma membrane integrity of sperm

Replication	Treatment					
	Upper layer			Lower layer		
	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>
.....%.....			.....%.....			
1	55.00	54.42	53.64	5.00	3.00	3.00
2	50.75	49.41	41.96	4.00	4.00	3.00
3	53.00	47.39	46.40	4.00	4.00	3.00
4	50.00	46.00	43.50	4.00	4.00	3.00
5	57.50	55.50	53.50	4.00	3.00	4.00
6	63.21	53.75	52.70	4.00	3.00	4.00
Total	329,46	306.47	291.70	25.00	21.00	20.00
Average	54.91±4.91	51.08±4.00	48.62±5.31	4.17±0.41	3.50±0.55	3.33±0.52

**Table 4.** Effect of incubation time during sperm sexing process on the abnormality of sperm

Replication	Treatment					
	Upper layer			Lower layer		
	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>
.....%.....			.....%.....			
1	4.50	5.50	4.50	4.50	4.00	5.00
2	5.00	4.50	4.50	4.00	4.50	4.00
3	4.50	4.50	5.50	6.00	4.00	4.50
4	3.00	4.00	4.00	3.50	4.50	4.50
5	3.50	3.50	4.50	4.00	4.00	5.00
6	3.50	3.00	4.00	3.00	3.50	4.50
Total	24.00	25.00	27.00	25.00	24.50	27.50
Average	4.00 ± 0.70	4.20 ± 0.80	4.50 ± 0.50	4.20 ± 0.90	4.10 ± 0.30	4.60 ± 0.30

serum albumin is a granular protein (globular) with a molecular weight of 66 kDa, and has a composition of 20 amino acids, in terms of its amino acid content, BSA has a more complete content than plasma semen (Gadea 2003) and that BSA can maintain the condition of sperm of any environment, including during the incubation process. Li Y et al. (2008) stated that the composition and conformation of BSA consist of one BSA molecule in a single polypeptide chain with a molecular weight of about 66.000 g/mol<sup>2</sup>. Thus the complete content in the BSA can maintain the condition of spermatozoa in any environment, including during the incubation process, so that with any incubation length, BSA solution can minimize the risk of disability on the head or tail (Leach et al. 2011). This is related to the opinion of (Gosálvez et al. 2011), the condition of the imperfect sperm cell membrane will increase of the abnormality of sperm. The abnormality of sperm after incubation for 45, 60, and 75 minutes was still below

20% in all treatments. Generally, the abnormalities occur in the tail of sperm that inhibit movement and reduce sperm fertility, but as long as sperm abnormalities have not reached 20%, the semen can still be used for artificial insemination (Alawiyah & Hartono 2006; Gosálvez et al. 2011).

**Effect of incubation time on DNA integrity of sperm after sexing**

The average DNA integrity of sperm after separation using BSA is presented in Table 5. Results of the statistical test showed that the incubation time did not significantly different on the DNA integrity in the upper and lower layer. This is expected because the males are superior males that have been selected and maintained to a good standard so that the good quality semen is produced. Environment and feeding are very

influential during the process of spermatogenesis, especially in the phase of spermiogenesis. Besides, the DNA position is located inside the sperm.

Sperm themselves have a protective membrane, namely the plasma membrane, the outer membrane of the acrosome as well as the inner membrane of the acrosome. In this study, membrane damage due to incubation time tends to be less, because the sexing method used is BSA, where BSA itself can protect the

integrity of the membrane from different environmental conditions. This is in line with Susilawati et al. (2014) which states that centrifugation can lead to damage cell membranes of sperms. Damage or a decrease in DNA integrity occurs when there is damage to the layers above, which will affect the integrity of DNA spermatozoa. This is thought to be a background factor in why the length of incubation does not affect the percentage of DNA spermatozoa integrity.

**Table 5.** Effect of incubation time during sperm sexing process on DNA integrity of sperm

Replication	Treatment					
	Upper layer			Lower layer		
	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>
	.....%......			.....%......		
1	98.00	97.00	97.00	99.00	97.00	98.00
2	99.00	98.00	98.00	97.00	98.00	98.00
3	98.00	98.00	98.00	98.00	98.00	98.00
4	99.00	99.00	99.00	98.00	98.00	98.00
5	99.00	99.00	99.00	98.00	98.00	97.00
6	99.00	98.00	98.00	99.00	99.00	98.00
Total	592.00	589.00	589.00	589.00	588.00	587.00
Average	98.66 ± 0.47	98.16 ± 0.68	98.16 ± 0.68	98.16 ± 0.68	98.00 ± 0.57	9.83 ± 0.37

**CONCLUSION**

It is concluded that the incubation time significantly affected the longevity of the sexed sperm of Pasundan bull, but not on plasma membrane integrity, abnormality, and DNA integrity.

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