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Use of Glycerol as Cryoprotectants in Freezing Sentul Chicken Semen

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Abstract

On the freezing semen chicken cryoprotectants required to overcome the damage of spermatozoa due to cold shock. This study aims to get the best concentrations of cryoprotectants glycerol concentration of 5%, 7% and 9% in freezing Sentul chicken semen. The semen used in this study came from three chickens Sentul and be repeated nine times. Semen was collected by messase methods for three times a week. Semen was evaluated macroscopic and microscopic. Furthermore spermatozoa diluted with egg yolk and the addition of three concentrations of cryoprotectants glycerol (5%, 7% and 9%). Semen diluted 0:25 ml is packed into straw. Then equilibrated at a temperature of 5°C for two hours. After equilibration to evaluate the motility and viability of spermatozoa. Furthermore, frozen in liquid nitrogen vapor for 10 minutes. Frozen semen is then stored in liquid nitrogen containers with temperature -196°C. After 24 hours, semen is thawed at 37°C for 30 seconds. The results showed that the percentage of sperm motility and viability of frozen semen cock Sentul using glycerol cryoprotectants 5% better P (<0.05) compared with the use of glycerol 7% and 9%. The use of glycerol 5% at this stage of equilibration and storage can reduce the damage of spermatozoa in the semen of chicken Sentul. Neither glycerol 5% could increase recovery rate after thawing.

Keyword: frozen semen, chicken Sentul, glycerol

A. Introduction

The chicken is a commodity cattle that is very common in Indonesia. A genetic resource of local chickens known to have a genetic variation is quite high. One type of local chicken is chicken Sentul among which is the original chicken Ciamis regency endangered and is now intensively maintained by several groups Sentul chicken lovers. In the breeding program of male sperm quality testing needs to be done to get high productivity. Semen that has led to poor quality eggs fertility is low and vice versa for good quality semen will produce a percentage of fertile eggs better. It depends on the stud, in particular the quality of the semen produced by stud.

One effort Sentul chicken germplasm conservation is the conservation of semen. Cryopreservation of semen is one of the common ways to extend the viability of spermatozoa

and one of the ways to preserve endangered germplasm chicken. Cryopreservation techniques as well as an additional supporting factor is excellent for in situ conservation and is also used in the selection program. Meanwhile, according to Fulton (2006) cryopreservation of germplasm will be a very valuable tool for the poultry industry and for the successful preservation of poultry genetic resources that still exist.

In the freezing process frequently encountered is cold shock and cell damage caused by the formation of ice crystals. Thus it is critical to the success of the poultry semen cryopreservation cryoprotectants with the election of the right to protect spermatozoa from freezing effect (Suidzinska and Lukaszewicz, 2008). Diluents semen should contain a source of nutrients, buffers, anti cold shock, cryoprotectants, antibiotics, easily soluble in water and must have a molecular weight small in order to more quickly penetrate into cells and reduced toxicity due to osmolarity is high (Alvarenga et al., 2005). To overcome the cold shock and the formation of ice crystals, then in the process of freezing semen cryoprotectants added to stabilize the plasma membrane of spermatozoa during the freezing process. Cryoprotectants role in protecting spermatozoa during cryopreservation (Arifiantiniand Supriatna, 2007).

Glycerol is intracellular cryoprotectants are most widely used for freezing semen. Glycerol can go inside sperm cells to bind most of the free water, so that ice crystals are formed in the diluent medium at the time of freezing can be prevented. The concentration of glycerol used is different depending on the type of semen and diluent used (Azizah and Arifiantini, 2009).

Cryoprotectants are needed to prevent the formation of ice crystals, but also is toxic cryoprotectants during equilibration and after thawing. On the basis of research conducted with the use of cryoprotectants glycerol level of 5%, 7% and 9% to get the best of glycerol concentration on freezing semen on chickens Sentul as local chicken Indonesia.

B. Methodology

1. Materials

Sentul male chickens used as a source of semen in this study have sex adult, 1 year old, able-bodied and have the best quality of daily semen production. Sentul male chickens caged individually. Feed given in the form of finished feed (commercial feed) shaped pellets of companies PT. Gold Coin Indonesia. Feed nutrient content is at least 17% crude protein; 2229.40 Kcal of energy metabolism, a maximum of 6% crude fiber, at least 3% fat, up to 14% Ash, 0.6-1% phosphorus, and calcium 3.0-4,2%. Feed given at a dosage of 150 g / day and the provision of drinking water adlibitum. Chickens used Sentul male semen collector and adapted to the cage environment. Adaptation takes two months. Once accustomed to the cage environment and collectors, the new chicken can be used in research to be accommodated semen.

2. Method

This research was conducted in cages Poultry Breeding, Faculty of Animal Husbandry and Animal Reproduction Laboratory, Faculty of Veterinary Science IPB. The study was designed using completely randomized design (CRD) with three treatments cryoprotectants glycerol (5%, 7% and 9%) and nine Deuteronomy.

Preparation Materials diluents

Diluent used is glucose, egg yolks, penicillin, streptomycin, glycerol and ringer lactate. Concentration Glycerol is used as a diluent (Table 1).

Table 1. Composition of Diluent Used Frozen Semen

Composition	Glycerol		
	5 %	7 %	9 %
Ringer lactate (mL)	8.5	8.3	8.1
Yolk (mL)	1	1	1
Glycerol (mL)	0.5	0.7	0.9
Penicillin (IU mL^{-1})	1000	1000	1000
Streptomycin (mg mL^{-1})	1	1	1
Total (mL)	10	10	10
Osmotic Pressure (mOsmol kg^{-1})[a]	1230	1800	2298
pH[b]	7	7	7

[a]Diluent osmotic pressure was measured using osmometer. [b]pH diluent adjusted (served) with*Tris hydroxymethyl aminomethan.*

The equipment used for the storage of semen and evaluation of both semen fresh or frozen semen is the syringe 1 cc, microtube 2 cc, a light microscope electricity (Olympus CH 20), microscopes, glass objects, glass cover, tube rack semen, counting chamber, plastic pipette, straw cutter or scissors, and refrigerators. While the equipment Involved to kriopresevasi semen Including freezing and thawing of frozen semen include collection tubes semen 2 cc, test tubes 5 cc, micropipette, tip micropipette, styrofoam box, set the storage of frozen semen (storage container) or container of liquid nitrogen (- 196oC), pipette, straw-sized 0:25 ml, 1 cc syringe fitted with connectors straw, tube racks, semen in a plastic box, Bunsen, a thermometer and a fridge.

Semen collection Fresh Chicken Sentul

Sentul chicken semen collection from each treatment three times a week. The technique used is semen shelter with sorting (massage) on the back of the chicken. Shelter semen carried by two people. Holding a chicken on his thighs with his left hand while massaging the back of the chicken to stimulate the release of semen, and another preparing large-scale semen reservoir tube and a tissue cleanser chicken manure. Ordering is done several times until the occurrence of a stimulus in chickens characterized by stretching the chicken's body and the release of proktodaeum cloacal papillae. When the erection reaches a maximum, right and left hands of people who do the sorting cooperate flushed semen. At the same time, the second man getting ready to accommodate large-scale semen reservoir tube.

Semen Evaluation Fresh Chicken Sentul

Fresh semen evaluation is performed to determine the quantity, quality and characteristics of fresh semen from a male chicken Sentul. Semen examination carried out according to standard methods which include macroscopic and microscopic evaluation.

a. Macroscopic Evaluation

Microscopic evaluation conducted to estimate the quality of semen in plain covering;

1. Volume: Total volume per ejaculation, can be directly observed on the scale tube.
2. Color: The color is observed in organoleptic through direct observation.
3. Consistency / degrees of viscosity: done in a bright place, by way of the tube is tilted and a few moments later reestablished. Scale viscosity of semen is observed that liquid (semen will soon return to the bottom of the tube, being (semen will soon return to the bottom of the tube at a speed that is slower than the first, most semen still attached to the wall of the tube) and thick (semen back to the bottom of the tube slowly and left semen on the outskirts of the tube.
4. pH is measured with a pH indicator.
5. Smell: can be determined by kissing on the surface of the tube.

b. Microscopic evaluation

1. Movement of spermatozoa
   a. Mass Movement (mass activity): It is an evaluation conducted to see the motility of spermatozoa to move together. How inspection and penilaan mass movement is dripping with semen to glass objects and then observed under a microscope with a magnification of 10 X 10 (100X), waves are seen in the form of a cloud-like clot. Assessment is done by looking at the thickness of the mass wave of spermatozoa and spermatozoa velocity change places. Ratings are determined from:
      1. Very good (+++) is a mass wave of thick and fast on the move.
      2. Good (++) is a thick mass wave but slow migratory or mass wave being but a quick move.
      3. Medium (+) is a mass wave of thin and slow move.
      4. Poor (O) or necrospermia (N) is no mass wave.
   b. Individual progressive movement / motility: progressive motility of spermatozoa by calculating the percentage of progressively motile spermatozoa of total spermatozoa in the sample. Because semen contains millions spermatozoa, the semen was diluted using physiological fluids (physiological NaCl 0.9%). As for the method of calculating the progressive motility of spermatozoa is subjective basis of five quantitative visual field using an electric light microscope (Olympus CH 20) magnification of 400 times in the range of 0-100% with a scale of 5%.

2. The percentage of spermatozoa (sperm count / ml of semen): How is the determination of sperm concentration, sperm diluted with formasalin much as 499 microns and semen as much as 1 micron then dribbled sperm that has been diluted to a glass object. The concentration was observed wearing a counting chamber is then observed under the microscope.
3. Percentage of Life (Viability): Performed by using the preparations and eosin staining nigrosin. Spermatozoa counted in 10 random visual fields, the head of spermatozoa which do not indicate that the spermatozoa stained and stained life is dead spermatozoa

$$\text{The persentage of live sperma} = \frac{\text{Number of Live Sperma}}{\text{Total Spermatozoa}} \text{x} 100\%$$

4. Sperm abnormalities: Abnormalities of spermatozoa obtained by calculating abnormal spermatozoa with making preparations pillowcase and observed under a microscope with a magnification of 10 x 40. As for the method of calculating the number of abnormal spermatozoa is abnormal spermatozoa divided by 200 multiplied by one hundred percent visible. The percentage of abnormal spermatozoa will be obtained by using the following formula :

$$\text{Abnormalities of Spermatozoa} = \frac{\text{Abnormalitas of Spermatozoa}}{200} \text{x} 100\%$$

5. Pressure Measurement Osmosis Fresh Semen Materials and diluents

Semen Packaging

Semen that has been qualified and then do the next process. The terms of motility that is more than 60%, the mass movement ++ / +++, the percentage of living at least 65%. Semen is diluted with the treatment of this type of cryoprotectants glycerol with a concentration of 5%, 7% and 9%, inserted into the straw.

Semen equilibration of Chicken Sentul

Furthermore straw that has been filled with semen was placed in the refrigerator for equilibration process, at a temperature of 5 ° C for 2 hours. Then half of the semen which has been equilibrated observation motility and viability (percentage of live sperm) to determine the decline in semen quality after equilibration. The rest of the Straw that has been equilibrated then placed on a shelf freezing, which placed 5 cm above the surface of liquid nitrogen for at -110oC temperature for 10 minutes.

Storage

Frozen semen stored in liquid nitrogen for further testing.

Semen Quality Testing Sentul Post-thawing Frozen Chicken

Tests carried out 24 hours after storage. Semen freezing-thawing in warm water (37 ° C) for 30 seconds. Parameters measured in semen examination are the percentage motility and viability. The success of freezing also assessed by Recovery rate (RR) is the percentage of spermatozoa was successfully recovered from the freezing process is calculated by comparing the percentage of motile spermatozoa in fresh semen, and after thawing.

$$\text{RR} = \frac{\text{The Persentage of Motile Sperma of ter Thawing}}{\text{The Persentage og Motile Spermatozoa Fresh Semen}} \text{x} 100\%$$

C. Result and Discussion

1. Characteristics of Semen Fresh Chicken Sentul

Macroscopic observation showed that semen quality fresh chicken Sentul fairly well (Table 2). All the characteristics of the fresh semen in the normal range. The degree of acidity (pH) is relatively neutral with the average of 0.12 ± 7:07, white, thick consistency, a great mass movement (+++) and a distinctive smell. As stated by Iskandar (2007) that the quality of good sperm should have a thick and creamy white. Although the average volume of chicken Sentul obtained only 0:12 ± 0:03 ml or lower than 0.2 ml volume Kampung chicken (Junaedi et al., 2016); 0:22 Pelung chicken ml (Junaedi 2015); and chicken Merawang 0:17 ml (Sartika, 2010). The low volume of research Sentul chicken due semen shelters often done in a week ie three times a shelter in a week. It shows that the semen shelters often do chicken can reduce semen volume.

Table 2. Characteristics of Semen Fresh Chicken Sentul

Parameter	Result
Color	White
Viscosity / consistency	Thick
Mass movement	+++
Smell	Typical
pH	7:07 ± 0:04
Volume (ml)	0:13 ± 0:01
Motility (%)	83.33 ± 1.86
Viability (%)	92.36 ± 1:51
Concentration (M/ml)	2956 ± 328.77
Concentration/Ejaculation (million/ ejaculation)	376.88 ± 36
Total Abnormalities (%)	6.87 ± 1:09
Primary abnormalities	
-Abnormalitas Acrosome (%)	1:11 ± 0.96
-Abnormalitas Head (%)	1:19 ± 0:29
abnormalities Secondary	
-Abnormalitas Weight (%)	0:08 ± 0:13
-Abnormalitas Tail (%)	4.73 ± 0.71
The osmotic pressure (mOsmol / kg)	277

Microscopic motility in Sentul chicken was 83.33 ± 1.86%. This figure is within the range of normal motility which ranges from> 70% according to the instructions Dumpala et al. (2006). Motility is one indicator of the size of the ability of spermatozoa fertilize the ovum in the fertilization process. Progressive motility of spermatozoa (move forward) become an absolute benchmark that counts. This means moving spermatozoa spinning or moving in a much less that do not move are not used as a measure of quality assessment of spermatozoa. Viability of fresh semen chicken Sentul 1:51 ± 92.36% larger than the chicken viability Parent stock of 82.3 ± 5.9% based on the results of research Castillo et al. (2010), chicken Pelung 90.97% (Junaedi 2015) and Kampung chicken 91.14% by Junaedi et al. (2016).

Sentul chicken sperm concentration obtained was 2956 ± 328.77 million / ml and this figure is lower than some other breeds of chickens are 3.12 billion chicken Kampung (Junaedi et al., 2016); 3.1 billion chickens Arab (Ervandi, 2011); 3:19 Pelung chicken billion (Junaedi 2015) and 3 billion in chickens Filina (Saleh, 2004). The concentration of spermatozoa / ejaculate chicken Sentul is 376.88 ± 36 million / ejaculation. The concentration per ejaculate in chickens is calculated to determine the ability of males to cradles females.

The total percentage of abnormal spermatozoa chicken Sentul 1:09 ± 6.87%. There are two kinds of abnormal spermatozoa were found in chickens Sentul i.e. primary abnormality includes abnormality acrosome 1:11 ± 0.96% and ± 0:29 1:19 head abnormality% while secondary abnormalities include abnormalities of body 0:08 ± 0:13 tail abnormalities 4.73% and ± 0.71%. In general, the primary abnormality factor that most fighting is a genetic disorder of the individual animals while secondary abnormalities were most instrumental factors are environmental factors. In general abnormality chicken spermatozoa abnormalities both in total and on primary and secondary abnormality is still in the low category.

2. Semen Quality Frozen of Chicken Sentul

Statistical analysis of variance showed treatment usage levels of cryoprotectants glycerol after equilibration significant effect (P <0.05) on motility of frozen semen spermatozoa chicken Sentul. The percentage of motility in the use of glycerol 5% (3:53 ± 78.33%) better than the glycerol concentration of 7% (71.77 ± 6.97%) and glycerol concentration of 9% (3:31 ± 73.51%). Based on these results it can be seen that the concentration of glycerol overload can cause toxic to spermatozoa so that the content of glycerol which leads to high motility of spermatozoa is low. While the viability of spermatozoa after equilibration in glycerol treatment showed no effect (P> 0.05).

Sentul chicken frozen semen quality after thawing is best assessed quantitatively is the use of glycerol 5% to 36.48 ± 4:28 motility and viability 50.08% ± 3.89% (Table 3). Based on statistical

analysis of sperm motility test on 5% glycerol treatment was significantly different p (<0.05), with 7% glycerol and glycerol 9%. The low percentage of motility and viability after thawing in chickens because spermatozoa are easily damaged. Poultry spermatozoa head shape like a crescent, causing the neck is broken in the process of storage. From these results, it is known that excessive use of glycerol which can degrade the quality of frozen semen. It is also explained by Ihsan (2013) that the metabolic processes spermatozoa will produce energy and a bit of lactic acid, but when the concentration of glycerol in excess diluent / too high, it will form more of lactic acid. Lactic acid can lower the pH and is toxic to spermatozoa.

Table 3. Quality of freezing semen at the various stages of the three concentrations of glycerol

stages freezing	Glycerol Concentration (%)		
	5	7	9
Fresh Semen			
• Motility (%)	83.33 ± 1.86	83.33 ± 1.86	83.33 ± 1.86
• Viability (%)	92.36 ± 1:51	92.36 ± 1:51	92.36 ± 1:51
After Equilibration			
• Motility (%)	78.33 ± 3:53[a]	71.77 ± 6.97 [b]	73.51 ± 3:31 [b]
• Viability (%)	88.12 ± 3:57	87.17 ± 3.75	85.61 ± 3:15
After *Thawing*			
• Motility (%)	36.48 ±4:28 [a]	30.92 ± 8.90 [b]	31.78 ± 8:53 [b]
• Viability (%)	50.08 ± 3.89	47.06 ± 9.94	43.53 ± 9:57
Recovery Rate (Rr)	44.03 ± 6:51[a]	36.95 ± [ab] 10:17	33.54 ± 11.72 [b]

Description: different letters that follow the numbers on the same row and column shows the difference (significantly different (P <0.05)), while the results are not contained letters show results not significantly different (P> 0.05).

The success is not only seen freezing semen quality after thawing, but also from the number of spermatozoa were successfully recovered from the freezing process called recovery rate (RR). RR value of the use of diluent glycerol 5% higher (44.03 ± 6:51%) compared with 7% glycerol (36.95 ± 10:17%) and glycerol 9% (33.54 ± 11.72%). In the statistical analysis showed that 5% glycerol significantly different (P <0.05) with glycerol 9% but not significantly different from the concentration of glycerol 7%. Glycerol will provide effective protection against spermatozoa during the freezing process if the concentration is in the optimum dilution.

Osmotic pressure test shows that fresh semen on chickens Sentul has a value of 277 mOsmol/kg (Table 2). Osmotic pressure testing is useful for determining the exact concentration of glycerol used in chicken semen freezing. The test results osmotic pressure on diluent semen is 1230-2298 mOsmol/kg (Table 1) shows the osmotic pressure is considerably higher than the osmotic pressure of semen chicken. The test results osmotic pressure diluent with the addition of a diluent of glycerol obtained semen frozen chicken with ideal osmotic pressure is 1230 mOsmol/kg. The ideal osmotic pressure obtained from the addition of 5% glycerol concentration in the frozen semen diluent. Based on these results it appears that diluent has hiperosmotik pressure. The high osmotic pressure outside the cell will cause the release of water from the cells, causing the cells to contract and the cell fluid that comes out is replaced by cryoprotectant (Souhoka *et al.*2009).

3. The decline in the quality of spermatozoa Semen Frozen Chicken Sentul

a) Decrease in Motility

To facilitate the evaluation of the success and know the quality of each stage of the decline in this study, we evaluated the motility of fresh semen, after equilibration, and after thawing. The decline in the percentage of motility in the study of fresh semen - after equilibration - after thawing results showed that the use of glycerol 5% is the best. 5% glycerol to minimize the loss of quality of spermatozoa at each stage (Figure 1). Deterioration in the quality of fresh semen to after equilibration is low ranging between 5-11.56%, from after equilibration after thawing decline to high start between 40.85 - 41.85%. While overall that the decline in the quality of fresh semen-spermatozoa after thawing ranged 46.85-52.41%.

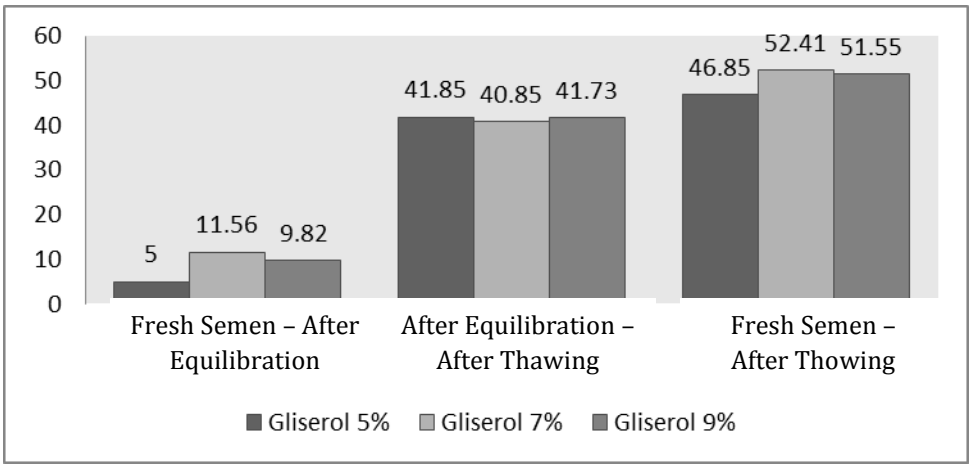

Figure 1. The decline in the percentage of sperm motility Sentul chicken at every stage clotting

b) Decrease in Viability

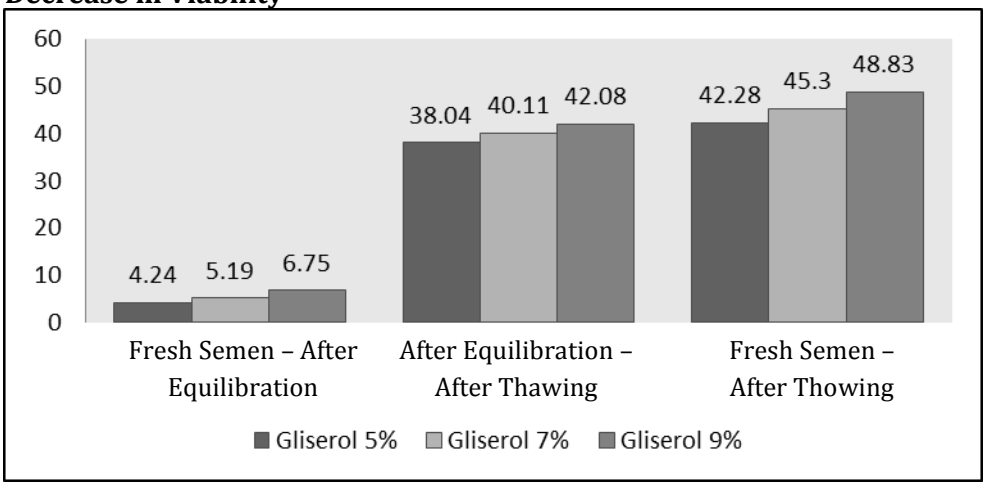

Figure 2. The decrease in the percentage of spermatozoa viability Sentul chicken at every stage clotting

From the results of research on semen frozen chicken Sentul with the use of three concentrations of glycerol was found that the decline in the quality of spermatozoa from fresh semen after equilibration obtained the best results in a row glycerol 5% (4:24%), glycerol 7% (5:19%), then glycerol 9% (6.75%), while after equilibration best account after thawing glycerol consecutively glycerol 5% (38.04%), glycerol 7% (40.11%) and glycerol 9% (42.08%). The quality of spermatozoa from fresh semen - after thawing use of glycerol 5% (42.28%) and glycerol 7% (45.3%) and glycerol 7% (48.83%). Loss of viability of spermatozoa occurs because the chemical properties that produce spermatozoa cell metabolism which can be toxic to life. Yulnawati and Setiadi (2005) explains that the spermatozoa are dead and become toxic to spermatozoa were still alive, so in general a quality decrease. The existence of substances that are toxic both derived from the spermatozoa that have died or are derived from substances contained of a diluent which has undergone oxidation due to storage can lead to high levels of free radicals that can damage the integrity of the plasma membrane of the spermatozoa. Neither the opinion Solihati et al. (2006) that the number of dead spermatozoa will affect the spermatozoa were still alive during the storage process.

D. Conclusion

From the research that has been done can be concluded that the use of glycerol 5% at this stage of equilibration and storage can reduce the damage of spermatozoa in the semen of chicken Sentul. Neither the addition of 5% glycerol concentrations can increase recovery rate after thawing.

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