The Abnormality of Spermatozoa Goat after Freezing on -80°C Using Tris Diluent Added Combination Hatching Egg Yolk and Amniotic Fluid

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Abstract

This study aims to determine the combination of the hatching egg yolk (HEY) and amniotic fluid (AF) in Tris diluent to the type of goat spermatozoa abnormalities after frozen at temperature -80°C. This study used four treatments and 6 replications. The semen is diluted using tris extender and added treatment, there are control group (T_0) (Tris egg yolk 20%), T_1 (15% HEY + 5% AF), T_2 (10% HEY + 10% AF), and T3 (5% HEY + 15% AF). After dilution, the semen was equilibrated for 2 hours, then stored using *Mr. Frosty* and frozen at temperature -80°C in the ultra-freezer for 24 hours. The type of spermatozoa abnormalities type was evaluated on fresh semen and after thawing. The data were analyzed using ANOVA ($P<0.05$). The results showed that there is no significant difference among treatments. The primary abnormalities in fresh semen, there were macro-cephalic, amorphous, and the secondary spermatozoa abnormalities such as detached head, mid-piece defect, coiled tail, loosehead, shoe-hook tail, and a broken tail. The secondary abnormalities in post-thawing were found both in the control group and treatment, which are detached head, loosehead, coiled tail, shoe-hook tail, and broken tail. The conclusion of this study was the percentage of secondary abnormalities after thawing was increasing both in the control and treatment groups.

Keywords: Amniotic Fluid, Hatching Egg Yolk, *Mr. Frosty*, Type of Spermatozoa Abnormality.

INTRODUCTION

The advancement of biotechnology in livestock reproduction is very useful to increase the quality and quantity of livestock. Reproduction biotechnology that is widely developed, is artificial insemination technique [1]. Artificial insemination generally uses frozen semen from cryopreservation [2]. Cryopreservation generally uses liquid nitrogen. However, the availability of liquid nitrogen in some areas cannot be obtained easily [3], and the price is expensive. To overcome this obstacle, it is necessary to find an alternative way to replace the freezing media by freezing in the ultra-freezer. The freezing and storage of semen in the ultra-freezer have some advantages than using liquid nitrogen. There are large semen storage capacity, shorter equilibration processes, easy sample handling, no need to change the media places regularly, and the cost is cheaper [3].

During the cryopreservation process, the spermatozoa are susceptible to damage due to cold shock and ice crystals formation [4]. Previous studies showed the quality of spermatozoa post-thawing was decreasing. One of the most common parameters for examining the quality of spermatozoa is the morphology. Based on the previous study showed the percentage of post-thawing spermatozoa abnormality was increasing. This is caused by the changing of osmotic pressure in diluents [1,5,6].

To prevent the low quality of frozen semen, it needs a diluent that can maintain the quality of spermatozoa during storage. Commonly the used diluent has main components which contain nutrient, buffer, cryoprotectant, and antibiotic [7]. Another diluent component that is usually added is egg yolk because it has a role as an anti-cold shock component. Egg yolk has a composition of lipoproteins that can serve as extracellular cryoprotectant [8].

In this study, the diluent was added by amniotic fluid and egg yolk from chicken embryo in 8-day incubation. This based on the pH in egg yolk from 8-day incubation has a neutral pH (6.92) than fresh egg yolk [9] and it is suitable for addition to the diluent because goat semen has a normal pH (6.8-7.0) [1,10]. Amniotic fluid from chicken embryo has several amino acids such as histidine, serine, arginine, glycine, aspartame acid, glutamic acid, threonine, alanine, proline, lysine, tyrosine, valine, isoleucine, leucine, phenylalanine, methionine and various proteins [11]. Amino acid administration can prevent damaging during pre-freezing and post-thawing [12]. This study aims to determine the abnormalities of goat spermatozoa after freezing at -80°C using tris diluent which added

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with the combination of egg yolk from 8 days incubation and amniotic fluid from chicken.

MATERIALS AND METHODS

Research Material

Three healthy male goats between the age 1-2 years and the weight 88-93 kg were used in this experiment. Each goat was individually fed by *indigofera, odot* grass, and concentrate about 7-9 kg every day. Goats are maintained at Center for Artificial Insemination (BBIB) Singosari Malang. Semen goat was ejaculated using an artificial vagina and collected two times a week.

Hatching Egg yolk and Amniotic Fluid Preparation

Egg yolk and amniotic fluid were collected from six chicken eggs aged 8 days. The collection was conducted by using a syringe and stored at 4°C before used.

Research Methods

This study used 4 treatments and 6 replications each. The fresh semen was evaluated by the volume, concentration, pH, motility, viability and abnormality type. Fresh semen was diluted using tris diluent [13] (diluent A) composed of 2.96 g tris-aminomethane, 1.65 g citric acid, 2 g fructose, 0.1 g of penicillin, 0.1 g of streptomycin and 80 ml of aqubides. The Hatching Egg Yolk (HEY) and Amniotic Fluid (AF) was added to the diluent with the following comparison:

T_0 (control group) : tris diluent + 20% egg yolks
T_1 (treatment group 1) : tris diluent + 15% HEY + 5% AF
T_2 (treatment group 2) : tris diluent + 10% HEY + 10% AF
T_3 (treatment group 3) : tris diluent + 5% HEY + 15% AF

The addition of diluent after calculating the concentration of spermatozoa, and we used 100 million.mL^{-1}. The diluted semen was equilibrated in cool top at 4°C for 2 hours and added with diluent B (diluent A + glycerol 6.5%). After equilibration, the sample was put into cryofial then stored in *Mr. Frosty*, and frozen at -80°C in ultra-freezer. The abnormalities of spermatozoa were observed after 24 hours of freezing. The spermatozoa abnormalities was observed based on the method of Susilawati [14].

RESULTS AND DISCUSSION

The Quality of Fresh Semen

The qualities of fresh semen were examined in a macroscopic and microscopic parameter. The macroscopic examination includes volume and pH. The microscopic examination includes concentration, individual motility, viability, and abnormality type. Table 1 shows the macroscopic and microscopic parameter of fresh semen.

Table 1. Macroscopic and Microscopic Parameter of Fresh Goat Semen

Parameter	Average ± SD
Volume (mL)	1.8 ± 0.75
Concentration (Million.mL^{-1})	2244 ± 0.43
pH	6.8 ± 0.40
Individual motility (%)	45.8 ± 3.76
Viability (%)	77 ±8.29
Abnormality	4.66 ± 0.55

Note: SD = Standard Deviation

The semen volume in this study is higher than the previous study which the semen of Peranakan Ettawah goat is 0.86 mL.ejaculate^{-1} [15] and 1.03 mL.ejaculate^{-1} [16]. The differences of semen volume that was produced by each male goat can be influenced by animal age, collecting frequency, and individual variation of livestock [17]. The pH of semen in this study is normal. The normal pH of semen was 6.4-7.8 [18]. The pH variation of semen in each individual can be influenced by the concentration of lactic acid produced by spermatozoa metabolism [7].

Individual motility of goat semen in this study is 45.8%. This semen is still can be used for further process because the previous study which using motility 43% showed that the post-thawing motility is 15-26% [19]. The post-thawing motility 20% still can be used for insemination [20].

The viability in this study is 77% and this semen is categorized as good semen. It is based on the statement of Ducha et al. that state the viability cell is >70% is categorized as good semen and can be used for further processing [21].

The abnormality is 4.62%, and this percentage is lower than the semen in the previous study that the abnormality is 9.57% from Ettawah crossbreed goat [15]. The semen that used in this study is categorized as good semen because it is based on the statement of Hafez that the abnormality <20% indicates it is as good semen [22]. The observation of spermatozoa abnormality is necessary because it is associated with spermatozoa fertility, if the number of spermatozoa abnormalities is high then the spermatozoa's fertility will decrease [23].

The Spermatozoa Abnormalities in Post-thawing

The percentage of spermatozoa abnormalities after added by tris diluent combination egg yolk hatching and amniotic fluid in post-thawing evaluation can be seen in table 2. The statistical test of the spermatozoa abnormality in this study shows no significant difference in each treatment. The percentage of spermatozoa abnormalities after thawing in this study is lower than the previous study is 16-34% in Ettawah crossbreed goat [24], and 8.4-13.9% in Boer goat [25].

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Table 2. The Percentage of Spermatozoa Abnormalities in Post-Thawing

Treatment Group	Post-thawing Abnormality (% ± SD)
T_0	7.36 ± 1.47
T_1	5.86 ± 0.75
T_2	7.00 ± 2.00
T_3	8.83 ± 3.06

Notes:
SD = standard deviation
T_0 (control group) = tris diluent + 20% egg yolks
T_1 (treatment group 1) = tris diluent + 15% egg yolk + 5% amniotic fluid
T_2 (treatment group 2) = tris diluent + 10% egg yolk + 10% amniotic fluid
T_3 (treatment group 3) = tris diluent + 5% egg yolk + 15% amniotic fluid

The result of this study indicates that there is an increase in spermatozoa abnormalities from fresh semen to post-thawing. Treatment group 1 showed the percentage of spermatozoa abnormalities is lowest compared to the other treatment groups. The increased spermatozoa abnormalities after thawing may be caused by cold shock during freezing-thawing [22]. The other factors that cause increased abnormality are the temperature changes extremely and the dilution is not isotonic.

Hatching egg yolk contains lipoprotein and lecithin that can act as an extracellular cryoprotectant. Lipoprotein and phospholipid will cover the surface of the spermatozoa membrane by maintaining the phospholipid bilayer configuration of the spermatozoa membrane [26,27]. Thus, cold shock can be prevented during the cryopreservation process. Amniotic fluid contains amino acids that can act as antioxidants such as proline, histidine, glycine, and alanine. Based on the previous study, the addition of proline to the diluent can maintain the quality of horse spermatozoa during the cryopreservation process [28]. Amino acids can act as cryoprotection in keeping the spermatozoa membrane from the damage during freezing and thawing, and also protects spermatozoa during calcium uptake [29].

In the treatment group 3 (T_3) shows the highest percentage of abnormality is 8.83%. This is caused in 5% hatching egg yolk has fewer lipoprotein and lecithin so it's less optimum to act as extracellular cryoprotectant and buffer. This causes a decrease in membrane integrity in this treatment group. The composition of egg yolk in Tris diluent which can maintain the quality of spermatozoa is 10-20% [30].

Type of Fresh Semen Abnormalities and Post-Thawing

Spermatozoa abnormality was observed after the sample was stored inside the ultra-freezer at temperature -80°C for 24 hours. The types of spermatozoa abnormalities in fresh semen and post-thawing can be seen in Table 2.

Spermatozoa abnormalities are classified into two type, namely primary and secondary abnormality. Primary abnormality can occur during spermatogenesis in the seminiferous tubules. The type of primary abnormalities such as pyriform, tapered head, undeveloped, abnormal contour, narrow, macrocephalus, microcephalus, double head, and double tail [7,14]. The secondary abnormality occur when the spermatozoa through the epididymis, ejaculation, and processing sample during cryopreservation [31]. Secondary abnormalities such as midpiece droplets, loss of acrosome cover, tail folded, broken tail and coil tail [32].

Table 2. Types of Spermatozoa Abnormalities in Fresh Semen and Post-Thawing

Abnormality type	Fresh semen (% ± SEM)	Post thawing (% ± SD)			
		T_0	T_1	T_2	T_3
Tapered head	-	0.33 ± 0.51	0.50 ± 0.54	-	-
Detache head	0.33 ± 0.51	1.66 ± 1.21	1.83 ± 0.75	3.16 ± 0.30	3.16 ± 1.72
Macrocephalic	0.16 ± 0.40	-	-	-	-
Pyriform head	-	-	0.16 ± 0.40	-	-
Amorphous	0.16 ± 0.40	0.16 ± 0.40	0.50 ± 0.83	0.16 ± 0.16	0.16 ± 0.40
Double tail	-	-	-	-	0.16 ± 0.40
Droplet midpiece	0.66 ± 0.81	0.50 ± 0.54	-	-	0.16 ± 0.40
Coiled tail	1.66 ± 1.03	1.33 ± 1.50	0.66 ± 1.21	1.00 ± 0.81	1.00 ± 1.26
Bent tail	1.33 ± 0.81	1.66 ± 1.50	1.16 ± 1.47	0.83 ± 0.47	2.83 ± 1.32
Shoehook tail	0.16 ± 0.40	1.00 ± 0.89	0.20 ± 0.40	1.00 ± 0.25	0.33 ± 0.51
Broken tail	0.16 ± 0.40	0.33 ± 0.81	0.16 ± 0.40	0.50 ± 0.34	0.33 ± 0.51
Loose head	-	0.16 ± 0.40	0.50 ± 0.83	0.33 ± 0.21	0.66 ± 0.81
TOTAL	**4.66 ± 0.55**	**7.36 ± 1.47**	**5.86 ± 0.75**	**7.00 ± 2.00**	**8.83 ± 3.06**

Notes:
SD = Standard Deviation
T_0 (control group) = tris diluent + 20% egg yolks
T_1 (treatment group 1) = tris diluent + 15% egg yolk + 5% amniotic fluid
T_2 (treatment group 2) = tris diluent + 10% egg yolk + 10% amniotic fluid
T_3 (treatment group 3) = tris diluent + 5% egg yolk + 15% amniotic fluid

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In this study the primary abnormality in fresh semen such as detached head, macro-cephalic and amorphous. The secondary abnormality such as midpiece droplets, coiled tail, bent tail, shoe-hook tail and broken tail. According to Rodriguez-Martinez and Barth, the morphological abnormalities of spermatozoa are always found in every ejaculation [33].

The results of this study indicate an increase in secondary abnormalities after thawing. This result is supported by the previous study in human spermatozoa, there is an increase of abnormality in spermatozoa tail after cryopreservation [34]. The study of Ozkavucku et al. also showed an increased abnormality in detached head, loose head and coiled tail. Abnormality in the tail, especially coiled tail, are commonly caused by membrane damage, osmotic pressure and exposure by chemicals in diluents such as cryoprotectant glycerol [35]. When cryoprotectants replace the position of intracellular fluid it is causing changes in the composition of water during freezing, this causes coiled tail [36]. Another researcher suggested that the increase of spermatozoa abnormality post-thawing has the closely related to DNA damage. The cryopreservation process can cause the morphological change in spermatozoa and plasma membrane thus it influenced the chromatin structure spermatozoa [37,38].

CONCLUSION

The percentage of spermatozoa abnormalities was increased after thawing. Most of post-thawing spermatozoa abnormalities in the control group and treatment groups was a secondary abnormality. The spermatozoa abnormalities were found such as detached head, loosehead, coiled tail, shoe-hook tail, and broken tail.

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