PLASMA MEMBRANE AND ACROSOME INTACT OF TIMOR STAG SPERMATOZOA IN TRIS EGG YOLK DILUENT WITH DIFFERENT SOURCES OF CARBOHYDRATE STORED AT 3- 5°C

W M M Nalley[1], R Handarini[2], I Arifiantini[3] dan B. Purwantara[3]

[1]Faculty of Animal Science, Nusa cendana University Kupang, East Nusa Tenggara.
[2]Departement of Animal Science, Faculty of Agriculture North Sumatra University Medan
[3]Departement of Clinic, Reproduction and Pathology, Faculty of Veterinary Medicine, Bogor Agriculture University

Keywords : spermatozoa, intact plasma membrane, intact acrosome cap, timor stag

Introduction

Plasma membrane is the outer membrane of spermatozoa, which was protected the organels in the cell, so it can be normally function. A variety of semen storage preservation techniques have been applied to cervid species, but few direct comparisons have been made between different diluents and processing methods within species, or for a particular method across species. Most diluents used successfully in deer have been adapted from the commonly used sugar-based tris and or citrate-buffered ones developed for other small ruminants, using egg yolk for protection against cold shock. The purpose of this research was to study the used of different source of carbohydrate (glucose, fructose and sucrose) to maintenance the intact plasma membrane (IPM) and intact acrosome cap (IAC) of timor stag spermatozoa stored at 3 – 5°C.

Materials and Methods

Five healthy Timor stags (*Cervus timorensis*), which are approximately 3- 5 years aged at hard antler stage with normal testes. Stags were sedated using a combination of 1 mg xylazine and 2 mg ketamin i.m. kg^{-1} body weight. Semen was collected using electro ejaculator. Each collected fresh semen was evaluated for percentage of IPM and IAC. Both of parameters was observed every 24 h. Osmotic resistance test or hypo osmotic swelling (HOS) test methods by Revell dan Mrode (1994) used to evaluate percentage of IPM. Sperms stained with triphan blue (Nagy *et al.* 1999; 2001). to evaluate the intact acrosome cap (IAC). Data means were analysed by completely randomized design (CRD) and the difference compared by the least significant difference test.

Results and Discussion

Volume of fresh semen was 2.06 ml, pH 7.03, yellow white until creamy in color and the consistency rabged from medium to thick. The mass movement between ++ to +++ and the sperm motility was 75.83%. The average of sperm concentration was 842.35 x 10^6 ml^{-1}, viability of sperm was 87.67%, sperm abnormality was 7.31%, IPM was 76.83% and IAC was 80.17%.

The success of semen storage depends on numerous factors which may be peculiar to each species and are optimized according to the type of semen to be preserved. In this research the results showed that IPM using TS extender (24.44%) higher than using TG and TS extender (14.78% and 4.00%, respectively)(Table 1).

Table 1. Average percentage of timor stags IPM using different source of carbohydrate in tris egg yolk (TEY) dilluent stored in 3 – 5 °C

Day	Carbohydrate in tris egg yolk (TEY) dilluent		
	Glucose	Fructose	Sucrose
1	76.83 ± 7.44^a	76.83 ± 7.44^a	76.83 ± 7.44^a
2	74.44 ± 5.81^a	74.00 ± 2.86^a	72.78 ± 2.80^a
3	72.33 ± 6.19^a	66.78 ± 6.15^a	68.89 ± 4.36^a
4	68.22 ± 5.53^a	62.00 ± 7.58^a	67.44 ± 5.53^a
5	62.22 ± 5.60^a	57.65 ± 9.97^a	63.67 ± 7.37^a
6	59.22 ± 6.63^a	51.00 ± 12.84^a	59.22 ± 7.55^a
7	55.22 ± 8.42^b	42.11 ± 5.98^a	56.44 ± 7.36^b
8	51.00 ± 8.86^b	39.44 ± 6.01^a	53.44 ± 8.36^b
9	45.56 ± 11.78^b	36.44 ± 7.50^a	45.67 ± 5.58^b
10	41.67 ± 12.94^b	31.67 ± 5.61^a	43.89 ± 6.41^b
11	36.78 ± 9.52^b	23.22 ± 11.98^a	43.00 ± 5.42^c
12	29.67 ± 10.45^b	18.00 ± 11.31^a	36.89 ± 5.46^b
13	27.78 ± 9.63^b	14.67 ± 10.69^a	32.11 ± 4.36^b
14	22.44 ± 14.90^b	4.00 ± 0.00^a	30.56 ± 3.06^c
15	14.78 ± 13.25^b	4.00 ± 0.00^a	24.44 ± 3.92^c

Values with different superscripts within each

PRELIMINARY STUDY OF LESSER MOUSE DEER OOCYTE *IN VITRO* MATURATION AND FOLLICLE CRYOPRESERVATION

I. Djuwita[1], Hamni[2], C. Nisa'[1] and S. Agungpriyono[1]

[1]Departement of Anatomy, Physiology and Pharmacology, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor 16680, Indonesia
[2]Department of Anatomy, Faculty of Veterinary Medicine, Syah Kuala University Aceh Indonesia

Keywords : lesser mouse deer, oocytes, follicles, *in vitro* maturation, cryoreservation

Introduction

The lesser mouse deer (*Tragulus javanicus)* (in Malay *pelanduk* or kancil, in Indonesia) has become an interesting model for ruminant study since it is the world smallest known ruminant animal. In Indonesia, its population has been decreasing because of human destruction and illegal hunting; therefore, being protected and classified as endangered animal class II. The existence of this species should be sustained and efforts for conservation should be done since this species belongs to Indonesia fauna biodiversity as remarkable natural wealth. Several studies on conservation of the lesser mouse deer have been carried out either *in situ* or *ex situ*; however, so far breeding activity has not been successful yet. Therefore, to support the *ex situ* breeding activity of the lesser mouse deer, several studies concerned with female reproduction should be carried out. In this preliminary study, ovaries collected from sudden death lesser mouse deers (delivered to our laboratory) have been used for in follicles cryopreservation and oocytes *in vitro* maturation in order to sustain optimal utilization (gamete banking) of follicles and to enable to study the oocytes development which is very difficult to be carried out *in vivo*.

Materials and Method

Two pairs of ovaries collected from sudden death lesser mouse deers (from Java area) were kept in 0.89% saline solution supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin, at 30-35°C. Two ovaries were used for in ovary tissues cryopreservation using vitrification method in Phosphate Buffer Solution (PBS) containing 40% ethylene glycol, 0.5M sucrose and 3% Bovine Serum Albumine (BSA, Sigma, USA) (Djuwita *et al*, 2000). After vitrification and thawing, ovarian tissue were were fixed 24 h in Bouin solution for 5um serially section followed by deparaffinized, rehydrated and stained with Hematoxilin-Eosin. The morphology and number of follicles were examined in every five sections. Follicles were counted only when the nucleolus are present.and classified according to their morphological stage of development.
Two other ovaries were slice using sterile sillete in glass petridish containing 2 ml PBS containing 0.3% BSA and 50µg/ml gentamycin sulphate (Sigma, USA). The oocytes collected were evaluated and washed three times with maturation medium, which consists of Dulbeco's Minimal Eargle Medium (DMEM) supplemented with 10% (v/v) new born calf serum (Sigma, USA), 0.01 mg/ml follicle stimulating hormone (FSH, Denka Pharmaceutical, Japan), 5µg/ml insulin, 10 µg/ml tranferin, 5 µg/ml selenium (Boehringer Mannheim, German) and 50ug/ml gentamycin sulphate. The oocytes were cultured in 100 µl drops of the maturation medium, covered with mineral oil (Sigma, USA). Oocytes maturation was done in 5% CO_2 incubator at 38.5°C, for 26 hours incubation.

Matured oocytes were evaluated using procedure as reported by Djuwita *et al* (1998). The cumulus cells layers of oocytes were removed by using 0.1% hyaluronidase. Denuded oocytes were stained using fluorencens Hoecht-Propidium Iodine followed by examining under fluorecents microscope. Evaluation of oocytes matured status after *in vitro* maturation was based on observation metaphase-II (M-II) and the presenced of polar body I.

Results and Discussion

Ovary Tissues Cryopreservation

Table 1 shows that follicles in all stages of development still can be obtained from the sudden death mouse lesser deer, although the number of those with morphologically normal is very low. However, there is a tendency that

row are significantly different (abc , P<05).

Intact acrosome cap (was 26.33%, higher than IAC in TG and TF extender were 15.44% and 4.00% (Table 2). IPM and IAC in sucrose extender showed better quality than sperm stored in glucose and fructose extender. Fructose in deer semen extender is not principal, because the content of fructose in timor stag seminal plasma was only 1.66 mg/ml (Nalley, 2006). Tris–fructose was used as the first diluents part in the two-step method of Fiser *et al.* (1987) and tris has also been included as a component in some lactose and saccharose-based diluents.

Table 2. Average percentage of timor stags IAC using different source of carbohydrate in tris egg yolk (TEY) dilluent stored in 3 – 5 °C

Day	carbohydrat in tris egg yolk (TEY) dilluent		
	Glucose	Fructose	Sucrose
1	80.17 ± 8.08^{a}	80.17 ± 8.08^{a}	80.17 ± 8.08^{a}
2	76.33 ± 7.15^{a}	76.33 ± 4.05^{a}	76.33 ± 3.25^{a}
3	72.56 ± 6.62^{a}	71.33 ± 4.71^{a}	69.78 ± 3.78^{a}
4	70.11 ± 6.21^{a}	64.89 ± 7.55^{a}	68.67 ± 4.49^{a}
5	63.67 ± 6.11^{a}	60 11 ± 7.73^{a}	66.33 ± 6.59^{a}
6	59.56 ± 6.12^{ab}	53.00 ± 11.81^{a}	62.00 ± 7.36^{b}
7	55.67 ± 8.77^{c}	44.78 ± 6.06^{a}	59.22 ± 8.09^{c}
8	52.78 ± 8.50^{b}	41.11 ± 6.65^{a}	55.22 ± 8.66^{b}
9	47.67 ± 10.32^{b}	38.78 ± 6.95^{a}	46.22 ± 5.24^{ab}
10	43.44 ± 14.49^{b}	33.00 ± 6.09^{a}	45.11 ± 5.98^{b}
11	38.11 ± 9.13^{b}	23.67 ± 6.89^{a}	42.56 ± 4.29^{b}
12	30.67 ± 9.32^{b}	19.00 ± 5.43^{a}	38.78 ± 2.79^{b}
13	29.11 ± 9.42^{b}	16.11 ± 10.28^{a}	34.00 ± 1.79^{b}
14	22.89 ± 14.34^{b}	4.00 ± 0.00^{a}	31.67 ± 1.72^{c}
15	15.44 ± 13.71^{b}	4.00 ± 0.00^{a}	26.33 ± 4.31^{c}

Values with different superscripts within each row are significantly different (abc, P<05).

The tris–glucose diluents elaborated by Salamon and Visser (1972), is widely used and recommended for the frozen storage of ram semen (Salamon, 1976; Evans and Maxwell, 1987). In our study the IPM and IAC was decreased at daily observation. IPM in TS, TG and TF extender were decreased about 3.74%, 4.44%, 5.20%, respectively. IAC in TS, TG and TF extender showed the same profiles, which was 3.74%, 4.44% and 5.20%, respectively. Intact plasma membrane is the outer membrane of spermatozoa, which was protected the organelles in the cell including acrosome contains several hydrolytic enzymes is involved in the fertilization process (Hafez and Garner, 2000). Sperm with intact plasma membrane, containing enzyme in the mid piece region like aspirant aminotransferase can be change ATP to ADP for maintenance the sperm motility (Colenbrander *et al.*, 1992). When semen was stored at a low temperature, care had to be taken not to subject the spermatozoa to cold shock. This caused irreversible changes in sperm cells cooled to temperatures close to 0°C. The detrimental effects of cold shock, were overcome either by gradual cooling of semen from room temperature, or by addition of lipid to the diluents. Sugars perform several functions in the diluents. They add osmotic pressure to the medium and act as cryoprotectants (Watson, 1979). A recent study (Woelders *et al.*, 1997) using trehalose and sucrose, demonstrated a significant interaction between cooling rates and the presence of sugars. In this study, an isotonic sugar medium where the Tris–citrate components were substituted with sucrose and trehalose was significantly superior to a Tris–citrate–egg yolk medium in preserving acrosome integrity.

Conclusion

1. Sucrose and glucose is the best carbohydrate supplement to maintain IPM and IAC of sperm timor stag was stored in 3 – 5°C.
2. IPM and IAC were decreased every 24 h (3.74%-5.20% and 3.92%-5.44% respectively).

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