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Studies on *In Vitro* Fertilization of Follicular Oocytes of Feline using Fresh and Frozen Epididymal Sperm

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고양이의 신선 및 동결 정소상체 정액을 이용한 체외수정에 관한 연구

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SUMMARY

본 연구는 고양이의 신선 및 동결 정소상체 정액과 정소상체 정액 성상과 및 동결보존 시의 생존성 및 난포란과 정소상체 정자의 체외수정 후 체외수정율과 분할율에 대해 조사하였다. 고양이 정소상체 정액의 정자농도는 3.25±0.75×10^6 cells/ml, 정자의 활력은 70.85±4.20%, 기형정자 수는 8.55±1.85%로서 대조군인 사출정액의 정자농도는 5.05±0.40×10^6 cells/ml, 정자의 활력은 90.24±455%, 기형정자 수는 4.20±0.50%와 비교할 때 정자농도와 활력은 낮았으며, 기형정자 수는 많았다. 고양이 정액과 tris-buffer로 희석한 정액을 20분간 배양했을 때 정자농도는 3.50±0.40×10^6 cells/ml, 정자활력은 75.50±2.55%, 기형정자 수는 6.75±0.58%로서 희석하지 않은 정소상체 정액의 성상에 비해 약간 높게 나타났다. Tris-buffer로 희석한 고양이 정소상체 정액을 동결 융해했을 때 생존율은 54.50±4.45, 활력은 47.50±6.40%로서 희석하지 않은 대조군의 생존율 74.50±6.25%와 활력 78.50±5.20%에 비해 현저히 높게 나타났다. 고양이의 난포란과 신선 및 동결 정소상체 정자를 수정시켰을 때 체외수정율과 분할율은 68.30±5.35%, 57.25±4.35% 및 48.65±4.95%, 35.65±4.75%로서 신선 정자에 비해 동결 정소상체 정자로 수정시킨 군의 분할율이 유의하게 낮았다.

(Key words : epididymal semen, fresh and freezing, IVF, cleavage rate)

INTRODUCTION

Domestic pets were breed with hyper-protein and hyper-energy feed and breed in narrow space. So there exist reproductive failure problem. Because of oligospermia, increasing number of abnormal sperm and sterility, the resolution has been requested urgently. Also, it is means of solve the reproductive failure through the frozen semen and also means of preserve the gene forever (Mann, 1964; Gunzel, 1986; Province et al., 1984; Davis et al., 1963; Foote, 1964). Harrop (1962) reported that the survival rate with post-thaw sperm was 40~50%. Seager et al. (1975) were the first to gain success in pregnancy and parturition of feline utilized with frozen semen. Rota et al. (1997) has been

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reported that addition of Equex STM paste to a Tris extender improves post-thaw viability, motility, acrosome integrity and longevity of frozen-thawed cat spermatozoa. Axner et al. (2004) has been reported that the percentages of spermatozoa with Equex STM treated frozen- thawed semen intact acrosomes and motility were 66.7±15.2%~83.9±15.5% and 59.0±13.7~60.5±19.2% respectively. Several cryoprotocols have been used for both ejaculated and epididymal cat spermatozoa (Platz et al., 1978; Hay and Goodrowe, 1993; Wood et al., 1993; Lengwinat and Blottner, 1994; Stackecki et al., 1994; Schafer and Holzman., 2000 and Zambelli et al., 2002) and are usually associated with extensive acrosome loss and reduced motility(Pope et al., 1991; Hay and Goodrowe, 1993 ; Lengwinat and Blottner, 1994). Lengwinat and Blottner(2003) have been reported that the cleavage rates were significantly lower for zygotes produced by frozen spermatozoa(25.3%) than for zygotes produced by fresh spermatozoa (40.7%). To summarize these reports, the majority has focused on ejaculated frozen semen and there are few reports on the viability of frozen-thawed epididymal semen and *in vitro* fertilization of follicular oocytes using fresh and cryopreserved epididymal sperm.

This study was carried out to investigate general characteristics and the viability after frozen-thawed of tris-buffer feline epididymal semen and the fertilization rate of oocytes using fresh and frozen epididymal sperm.

MATERIALS AND METHODS

1. Experimental Animals

Eighteen captured cats(10~20 months) were used in this experiment. Within 20 min after castration the cauda epididymidis were transferred to laboratory and utilized in experiment.

2. Collection of Epididymal Semen

After castration the cauda epididymidis were placed in 400 μl of PBS at 38℃ in an Eppendorf tube and the tissue was removed from the PBS with warm forceps, the sample was centrifuged at 700×g for 6 min and diluted with physiological saline and tris-buffer(1:3)(Table 1).

3. Freezing of Semen

1) Dilution of Semen

After the supernatant was removed. Spermatozoa pellets were diluted in two stages with the frozen

Table 1. Component of semen extender

Composition	Extender-1	Extender-2	Extender-2'	Tris-buffer
Tris-buffer(g)	2.4	2.4	2.4	2.4
Citric acid monohydrate(g)	1.4	1.4	1.4	1.4
Fructose(g)	0.8	0.8	0.8	0.8
Na-Benzylpenicillin(g)	0.06	0.06	0.06	0.06
Streptomycin sulphate(g)	0.1	0.1	0.1	0.1
Egg-yolk(ml)	20	20	20	
Glycerol(ml)	3	7	7	
Equex STM paste(ml)	0	0	1	
D.W.	100	100	100	100
pH	6.53	6.56	6.48	6.6
Osmotic pressure	760	1,400	1,380	260

diluents(Table 1). Semen was supplemented with 1.5 ml extender-1 at room temperature and diluted solution was cooled for 45 min at 4℃. Following cooling for 25 min, 1.5 ml extender-2 and extender-2' were supplemented at 4℃ and then mixed. The straw semen preserved after being frozen for 1 month was placed for 30 min at room temperature and then dissolved in a water bath at 37℃. In order to remove cryoprotectants, frozen semen was shaken upside down and tris-buffer was removed and dissolved. The dissolved semen sample was moved to a slide glass and observed by microscope and the viability, survival rate, and morphological test were examined by using sperm analyzer imaging system(SAIS Si-100).

4. Freezing and Thawing of Epididymal Semen

Confirmed dilution at room temperature and frozen up to 4℃ at 1℃/min and 4℃～−30℃ at 0.2℃/min, −30℃～−38℃ at 0.2℃/min. And immediately plunged into LN_2 and frozen by automatic cell freezing method(Cell Freezer Forma Co., USA). The straw preserved after frozen for 1 month was placed for 30 min at room temperature and dissolved in water bath at 37℃ and in order to remove cryoprotectants, shaken upside and down and transferred to tris-buffer and dissolved.

5. Fertilization and Maturation

1) Culture of Oocytes

Ovaries were transported to the laboratory in sterile physiological saline. Oocytes were collected from ovaries or collected after being preserved at 4℃ or in a salt-solution(1.5 M $MgCl_2$) for 4, 24, 48 hrs. In order to collect oocytes recovered from fresh ovaries, preserved ovaries were washed with 20℃ physiological saline. Collected oocytes were cultured with TCM-199 medium supplemented with 10%(v/v) FCS. Ten oocytes were transferred to 50 μl drops of maturation medium under mineral oil and cultured in a CO_2 incubator(5% CO_2, 95% air, 38℃).

2) Semen Preparation

Semen was collected from tail of epididymis. In order to remove seminal plasma of whole semen was diluted with physiological saline and Tris-buffer solution(1 : 3) and centrifuged 700 g for 6 min and removed supernatant. Sperm pellets were diluted with fertile Tyrode solution. Before being utilized in an experiment, examined sperm motility, the number of survival sperm and conducted a morphologic examination by using sperm analyzer. Over 80～85% viability sperm and the semen which was frozen by Kim(2001) were thawed in 25℃ water-bath for 2 min. And then 0.01 ml semen was mixed with 500 μl BO medium and swim-up for 15 min in a incubator and the 0.5 ml of the supernatant was centrifuged at 500 × g for 5 min. Removed supernatant and remained sperm pellets were treated with 0.6% BSA(Sigma, USA) and 20 μg of heparin solution and utilized in further experiments.

3) *In Vitro* Fertilization

After *in vitro* maturation for 24～28 hrs, ten oocytes were transferred to each droplet and semen suspension of capacitation-sperm inseminated with oocytes in medium droplets. Inseminated oocytes were cultured in a CO_2 incubator(5% CO_2, 95% air, 38℃) for 12～24 hrs.

6. Survival Test

The dissolved semen sample was moved to a slide glass and observed by microscope and the viability, survival rate, and morphological test were examined by using sperm analyzer imaging system (SQA-IIB, Israel). The oocytes obtained from IVF was treated with 0.2% hyaluronidase(Sigma, U.S.A.) for 1～5 min. After cumulus cells were removed, denuded oocytes were fixed in acetic acid : ethanol

(1:3) solution for 24 h and stained with 1% aceto-orcein or 10 ㎍/ml bisbenzimide(Hoechst 33342, Sigma, U.S.A.). The judgment of oocyte maturation *in vitro* was carried out depending on the criteria of fertilization and cleavage by investigation the stained oocytes or embryo development by *in vitro* culture in CO_2 incubator.

7. Statistical Analysis

The results were expressed by treatment as mean±S.D. For comparison of means, Duncan's multiple verification was performed using SAS package of General Linears Model(GLM) procedures(SAS Institute, 1996).

RESULTS AND DISCUSSION

1. General Characteristics of Epididymal Semen

The general characteristics of feline epididymal semen tested by semen analyzer are shown in Table 2.

The sperm concentration, motility and number of abnormal sperm of feline epididymal semen were $3.25\pm0.75\times10^6$ cells/ml, 70.85±4.20%, 8.55±1.85%, respectively. This results were lower than that control group($5.05\pm0.40\times10^6$ cells/ml, 90.24±4.55%, 4.20±0.50%), but abnormal sperm was higher than that control group(4.20±0.50%). This result was higher than Kim et al.(2004) reported that the viability and dead sperm number of canine epididymal semen were 47.5±4.64~52.5±5.82% and 27.5±4.88~35.5±7.45% respectively.

2. Characteristics of Tris-Buffer Diluted Epididymal Semen

The sperm of concentration and motility and abnormal sperm rates of feline epididymal semen diluted with tris-buffer and removed seminal plasma by centrifugation and preserved 37℃ were show in Table 3.

The sperm of concentration and motility and abnormal sperm rates of incubated for 20 min dilution of feline epididymal sperm with tris-buffer were $3.50\pm0.40\times10^6$ cells/ml, 75.50±2.55%, 6.75±0.58%, respectively. This was higher that of non-dilution epididymal semen. This results were similar with Boucher et al.(1985) reported that compared with control group(72.7%), the result was indicated significant motility when cultured frozen epididymal semen of seminal plasma removed group and glycerol seminal plasma removed complex group were 85.5% and 75.5%, respectively.

Table 2. General characteristics of feline epididymal semen dilute with saline

Epididymal semen	Sperm con. ($\times10^6$ cells/ml)	Motility (%)	Abnormal sperm(%)
Whole semen*	5.05±0.40	90.24±4.55	4.20±0.50
Feline	3.25±0.75	70.85±4.20	8.55±1.85

* whole semen : ejaculated semen.

Table 3. General characteristics of feline epididymal semen dilute with tris-buffer

Dilution of tris-buffer	Sperm con. ($\times10^6$ cells/ml)	Motility (%)	Abnormal sperm(%)
Whole semen*	5.05±0.40	90.24±4.55	4.20±0.50
Feline	3.50±0.40	75.50±2.55	6.75±0.58

* whole semen : ejaculated semen.

3. Viability of Frozen-Thawed Epididymal Semen

The viability rate when the feline epididymal semen were diluted with tris-buffer and removed seminal plasma by centrifugation and equilibrated with −1, −2 and −2' extender and frozen-thawed were show in Table 4.

The viability rate when frozen-thawed tris-buffer dilution feline semen by slow freezing were 54.50 ±4.45%, 47.50±6.40%, respectively. This was lower than that of control group of fresh semen (85.70±5.54%, 88.65±6.50%). Although there was no similar report, this result was similar with Harrop(1962) and Foote(1964) reported that the survival rate of feline ejaculated semen was 40~60%.

4. *In Vitro* Fertilization and Cleavage Rate of Oocytes

The *in vitro* fertilization rate and cleavage rate when fertilized feline follicular oocytes and fresh and frozen epididymal sperm were show in Table 5.

The *in vitro* fertilization and cleavage rate of follicular oocytes of feline using fresh and frozen epididymal sperm were 68.30±5.35%, 45.25±4.35% and 48.65±4.95%, 27.65±4.75%, respectively. This cleavage rate was significantly lower for embryos produced by frozen sperm. These results were significantly similar with Lengwinat and Blottner(2003) has been reported that *in vitro* developmental rate was significantly lower when fertilized with cat follicular oocytes and fresh and frozen epididymal sperm were 40.7% and 25.3% respectively. Feline embryos were indicated high *in vitro* developmental rate, but the cleavage was inhibited at 32 cell or morula stage.

CONCLUSION

This study was carried out to investigate general characteristics and the viability after frozen-thawing of tris-buffer feline epididymal semen and the *in vitro* fertilization rate of oocytes using fresh and frozen epididymal sperm.

1. The sperm of concentration and motility and abnormal sperm rate of feline epididymal semen were $3.25\pm0.75\times10^6$ cells/ml, 70.85 ±4.20%, 8.55±1.85%, respectively. This was lower than that of control group of motility and higher than that of control group of abnormal sperm.
2. The sperm of concentration and motility and

Table 4. Survival rates after frozen-thawed feline epididymal semen freeze by cell freezer

Freezing of epididymal semen	Sperm motility of frozen-thawed semen	
	Survival sperm(%)	Motility(%)
Whole semen(control)	85.70±5.54	88.65±6.50[a]
Feline	54.50±4.45	47.50±6.40[b]

* Values with different superscripts within column were significantly difference ($p<0.05$).

Table 5. *In vitro* fertilization and cleavage rate of feline follicular oocytes using fresh and frozen epididymal sperm

Epididymal sperm	No. of oocytes examined	No. of oocytes fertilized(%)	Cleavage rate(%)
Feline-fresh	40	68.30±5.35	57.25±4.35[a]
frozen	40	48.65±4.95	35.65±4.75[b]

* Values with different superscripts within column were significantly difference ($p<0.05$).

abnormal sperm rates of incubated for 20 min dilution of feline epididymal sperm with tris-buffer were $3.50 \pm 0.40 \times 10^6$ cells/ml, 75.50 $\pm$2.55%, 4.82$\pm$0.58%, respectively. This was higher that of non-dilution epididymal semen.

3. The viability rate when frozen-thawed tris-buffer dilution feline semen by slow freezing were 54.50$\pm$4.45%, 47.50$\pm$6.40%, respectively. This was lower than that of control group of fresh semen(85.70$\pm$5.54%, 88.65$\pm$6.50%).
4. The *in vitro* fertilization and cleavage rate of follicular oocytes of feline using fresh and frozen epididymal sperm were 68.30$\pm$5.35%, 57.25$\pm$4.35%, 48.65$\pm$4.95%, 35.65$\pm$4.75%, respectively. This cleavage rate was significantly lower for embryos produced by frozen sperm.

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