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The Effects of Preservation of Ovaries, Incubation Time and Oocytes with and without Cumulus Cells on Zona Penetration by Canine Sperm

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개 난소의 보존, 난자의 배양시간 및 난구세포 부착 유무가 난자내 정자의 침입율에 미치는 영향

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SUMMARY

본 연구는 개의 불임해결과 체외수정란을 생산할 목적으로 난소의 보존 및 난구세포의 부착 여부가 신선 및 동결 개 정자를 이용한 투명대 반응에 미치는 영향을 조사하였다.

1. 적출한 난소를 4℃와 salt에 각각 48시간 보존 후 회수한 난구세포 부착 난자와 나화난자의 정자침입율은 각각 62.5%, 37.5% 및 42.5% 및 22.4%로서 난소를 적출 후 곧 바로 회수한 난구세포 부착 및 나화난자 내 정자침입율인 93.3%와 56.7%에 비해 현저히 낮게 나타났다.
2. 적출한 난소를 4℃에 각각 4시간, 14시간 및 48시간 보존 후 회수한 난구세포부착 난자의 정자침입율은 각각 92.5%, 90.0%, 85.0%로서 신선 난소 난자의 정자침입율 93.3%에 비해 유사하거나 약간 낮게 나타났다.
3. 적출한 난소를 salt에 각각 4시간, 14시간 및 48시간 보존 후 회수한 난구세포부착 난자의 정자침입율은 각각 85.0%, 77.5%, 72.5%로서 신선난소로부터 회수한 난자의 정자침입율 93.3%에 비해 낮게 나타났다.
4. 적출한 난소를 4℃와 salt에 각각 24시간 보존 후 회수한 난구세포부착 난자의 체외수정율과 체외발생율은 각각 50.0%, 22.5% 및 40.0%, 15.0%로서 신선난소로부터 회수한 난구세포부착 난자의 체외수정율과 체외발생율 72.5%와 32.5%에 비해 낮게 나타났다.

(Key words : canine, 4℃ and salt-stored ovaries, sperm penetration)

INTRODUCTION

The number of domestic pets being raised has surpassed 3 million and the number of people raising them has surpassed 5 million. Therefore pet care was enhanced gradually. IVM/IVF experiment which utilized canine oocytes needed a lot of ovaries. It is very difficult to obtain ovaries one

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time and the majority of ovaries were concentrated during the summer. In order to solve that problem, if after preserving ovaries temporarily at 4℃ or in a salt-solution there was no influence to the survival rate of oocytes, we can get the better experimental results.

Generally, the IVM and IVF rates of canine oocytes were lower than those of other animals. The maturation rate of canine oocytes was only about 50~60%, and 60~70% of oocytes were *in vitro* fertilized. Freistedt et al.(2001), Spindler and Wildt(1999) reported that the *in vitro* developmental rate of canine oocytes was 20~30%. It was reported that season(Freistedt et al., 2001; Goodrowe et al., 1991), culture condition (Johnston et al., 1991), reproductive cycle(Freistedt et al., 1990) and morphology of oocytes(Wood et al., 1995; Goodrowe et al., 1991; Pope et al., 1997) can affect oocytes *in vitro* developmental rate. For small dogs of sterility treatment, which had difficulty in sterility or natural fertilization, it was very essential of artificial insemination and transplantation of oocytes. Currently, artificial insemination is executed. But unlike other animals, there were many difficulties when transplanting embryos in dogs. Specifically, it is difficult to secure the ovaries temporarily, and also the *in vitro* fertilization technique is difficult, compared to those of other animals. When the quantity of collected ovaries was little, after temporarily preserving them, and re-treated many ovaries. If those would be utilized in an experiment, it would be bring a lot of advantages to the experiment.

The objective of this study was to produce *in vitro* fertilized oocytes and solute canine sterile. This study was carried out to investigate the effects of the preservation of ovaries and oocytes with and without cumulus cells and incubation time on zona penetration by canine spermatozoa.

MATERIALS AND METHODS

1. Oocyte Collection and Culture

Canine ovaries were transported to the laboratory in sterile physiological saline. Oocytes were collected from fresh 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. Oocytes were sliced with surgical blade and suspended with m-PBS and then collected. 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 covered mineral oil and cultured in a CO_2 incubator(5% CO_2, 95% air, 38℃).

2. Semen Preparation

2nd fractional semen was collected by a digital message method. 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 x 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 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.

4. Zona Penetration Assay

Incubated oocytes were washed in PBS. To remove cumulus cells and loosely bound sperm, oocytes were transferred to 0.02% hyaluronidase (Sigma, USA) solution in a 4-well dish, held for 10 min at room temperature, then vortexed in 200 μL of 1 % sodium citrate for 3 min. To stain bound and penetrated sperm, oocytes were transferred to 1 mL of a 100 μg/mL solution of Hoechest 33258(Sigma, USA) for 20 min. Examination of bound and penetrated sperm heads were carried out at 400 × magnification under fluorescent microscope. The number of sperm remaining on or in the zona pellucida of each oocytes was recorded.

5. Statistical Analysis

The results were expressed by treatment as mean±SD. 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. The Sperm Penetration of Oocytes with Different Preservation of Ovaries

In order to investigate zona penetration on preservation of canine ovaries. The sperm penetration rate of oocytes with and without cumulus cells recovered from fresh, 4°C and salt-stored ovaries were as Table 1.

The sperm penetration rate of oocytes with and without cumulus cells recovered from 4°C and salt-stored canine for 48 hrs were 2.5%, 37.5%, 42.5% and 22.4% respectively. That was significantly lower than the sperm penetration (93.3%, 56.7%) of oocytes recovered from fresh ovaries of without any intact treatment. The sperm penetration of oocytes with and without cumulus cells recovered from 4°C and salt-stored canine ovaries for 24 hrs was lower than the sperm penetration of fresh canine oocytes, but it confirmed that oocytes recovered from 4°C and salt-stored canine ovaries can be utilized *in vitro* maturation and *in vitro* fertilization. This results was significantly higher than Gabriela et al.(2002), reported that the sperm penetration of oocytes with cumulus cells recovered from 4°C and salt-stored canine ovaries for 24 hrs was 20.6~50.4%. Also, the sperm penetration of canine oocytes with and without cumulus cells recovered from salt I-stored and salt II-stored for 24 hrs were 50.4%, 39.1%, 44.2%, 20.6% respectively.

Table 1. Sperm penetration of oocytes with and without cumulus cells recovered from fresh, 4℃ and salt-stored canine ovaries

Type of ovaries	Recovered oocytes	Cultured oocytes	Penetration rate(%)
Fresh			
Intact	30	30	28/30(93.3)[a]
Denuded	30	30	17/30(56.7)
4℃-stored			
Intact	40	40	25/40(62.5)[b]
Denuded	42	40	15/40(37.5)
Salt-stored			
Intact	42	40	17/40(42.5)[b]
Denuded	44	40	9/40(22.5)

[a,b]: Values within column with different superscript (p<0.05).

2. The Sperm Penetration of Oocytes Recovered from 4℃-Stored Ovaries

The sperm penetration of oocytes with cumulus cells recovered from fresh and 4°C-stored canine ovaries for 4, 24, 48 hrs were as Table 2.

Table 2. Sperm penetration of oocytes with cumulus cells recovered fresh and 4℃- stored canine ovaries for 4, 24, 48 hrs

Type of oocytes	Recovered oocytes	Cultured oocytes	Penetration rate(%)
Fresh	30	30	28/30(93.3)
4℃ stored			
4 h	40	40	37/40(92.5)
24 h	41	40	36/40(90.0)
48 h	42	40	34/40(85.0)

The sperm penetration of oocytes with cumulus cells recovered from fresh and 4℃-stored canine ovaries for 4, 24, 48 hrs were 92.5%, 90.0%, 85.0%, respectively. That was similar or a little lower than the sperm penetration(93.3%) of oocytes with cumulus cells recovered from fresh ovaries. After preserved at 4°C for 4~48 hrs, the sperm penetration of oocytes with cumulus cells was 85.0~92.5%. It will be possible to get a high sperm penetration of oocytes recovered from 4°C-stored ovaries, and so it can be utilized for an IVM/IVF experiment. This result was lower than the Gabriela et al.(2002) reported that when the sperm penetration of oocytes with cumulus cells recovered from fresh and 4°C-stored canine ovaries for 4, 24, 48 hrs were 94.9% and 92.2%, respectively.

3. The Sperm Penetration of Oocytes Recovered from Salt-stored Ovaries

The sperm penetration of oocytes with cumulus cells recovered from fresh and salt-stored canine ovaries for 4, 24, 48 hrs were as Table 3.

The sperm penetration of oocytes with cumulus cells recovered from fresh and salt-stored canine ovaries for 4, 24, 48 hrs were 85.0%, 77.5%, 72.5%, respectively. That was lower than the sperm penetration(93.3%) of oocytes recovered from fresh ovaries. Compare to the sperm penetration of oocytes recovered from fresh ovaries was a little lower. However, according to the situation, the method which collected the ovaries after preserving them in a salt solution is could be used. The preservation hour was different but, the above result was significantly higher than that of Gabriela et al.(2002), reported that the sperm penetration of oocytes with cumulus cells recovered from fresh and salt-stored canine ovaries for 24 hrs was 20.6~50.4%.

Table 3. Sperm penetration of oocytes with cumulus cells recovered from fresh and salt-stored canine ovaries for 4, 24, 48 hrs

Type of oocytes	Recovered oocytes	Cultured oocytes	Penetration rate(%)
Fresh	30	30	28/30(93.3)
Salt-stored			
4 h	41	40	34/40(85.0)
24 h	43	40	31/40(77.5)
48 h	45	40	29/40(72.5)

4. *In Vitro* Fertilization Rate of Canine Oocytes with Different Preservation Methods

In vitro fertilization rate of canine oocytes with cumulus cells recovered from fresh, 4°C and salt-stored ovaries for 24 hrs was as Table 4.

In vitro fertilization rate of canine oocytes with cumulus cells recovered from fresh, 4℃ and salt-stored ovaries for 24 hrs were 50.0%, 22.5%,

Table 4. *In vitro* fertilization rate of canine oocytes with cumulus cells recovered from fresh, 4℃ and salt-stored ovaries

Type of semen	Cultured oocytes	Fertilized oocytes(%)	Developed oocytes(%)
Fresh	40	29/40(72.5)	13/40(32.5)[a]
4℃-stored	40	20/40(50.0)	9/40(22.5)[b]
Salt-stored	40	16/40(40.0)	6/40(15.0)[b]

[a,b] : Values within column with different superscript ($p<0.05$).

40.0%, 15.0%, respectively. This result was lower than *in vitro* fertilization rate(72.5%, 32.5%) of fresh canine oocytes with cumulus cells. Compare to fresh ovaries, the sperm penetration of oocytes recovered from 4℃ and salt-stored canine ovary was a little lower. However, according to situation, the oocytes could be utilized in *in vitro* fertilization after temporally preserved. This result was similar or a little lower than Kim et al.(2004), reported that the *in vitro* fertilization rate(23.3~57.1%) and cleavage rate(23.3~3.3%) of salt-stored feline ovaries.

CONCLUSION

The study was carried out to investigate the effects of preservation of ovaries and oocytes with and without cumulus cells and incubation time on zona penetration by canine spermatozoa. The objective of this study was to produce *in vitro* fertilized oocytes and solute canine sterile.

1. Sperm penetration of oocytes with and without cumulus cells recovered from 4℃ and salt-stored canine ovaries for 48 hrs were 2.5%, 37.5%, 42.5% and 22.4% respectively. That was significantly lower than the sperm penetration (93.3%, 56.7%) of oocytes recovered from fresh ovaries of without any intact treatment.
2. Sperm penetration of oocytes with cumulus cells recovered from fresh and 4°C-stored canine ovaries for 4, 24, 48 hrs were 92.5%, 90.0%, 85.0%, respectively. That was similar or a little lower than the sperm penetration(93.3%) of oocytes with cumulus cells recovered from fresh ovaries.
3. Sperm penetration of oocytes with cumulus cells recovered from fresh and salt-stored canine ovaries for 4, 24, 48 hrs were 85.0%, 77.5%, 72.5%, respectively. That was lower than the sperm penetration(93.3%) of oocytes recovered from fresh ovaries.
4. *In vitro* fertilization rate of canine oocytes with cumulus cells recovered from fresh, 4°C and salt-stored ovaries for 24 hrs were 50.0%, 22.5%, 40.0%, 15.0%, respectively. This result was lower than *in vitro* fertilization rate(72.5%, 32.5%) of fresh canine oocytes with cumulus cells.

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