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In Vitro Fertilization and Development of *In Vitro* Matured Porcine Oocytes by Morphologically Normal Sperm Ratio of Epididymal Sperm and Frozen-Thawed Ejaculated Sperm

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정소상체 미부정자와 동결 융해된 사출정자의 형태학적 정상정자 비율이 체외성숙된 돼지난자의 수정 및 발달에 미치는 영향

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요 약

본 연구는 돼지난포란의 체외수정시 정소상체 미부정자의 형태학적 정상정자 비율에 따른 수정율과 배발달율을 조사하고자 실시하였다. 그 결과는 다음과 같다.

1. 정소상체 미부정자의 형태학적 정상정자 비율을 ≤10%, 10~30%, ≥50%로 구분하여 수정율과 배발달율을 조사하였던 바, ≥50%에서 각각 64%, 26%로 나타나 다른 두 군보다 현저히 높게 나타났다 (≤10%: 27%, 6%, 10~30%: 36%, 5%) ($p<0.01$).
2. 50% 이상의 정상정자를 가진 정소상체 미부정자를 100% (5×10^5 cells/ml)로 조정하여 수정하였던 바, 정소상체 미부정자군의 수정율과 배발달율 (63%, 27%)은 동결 융해된 사출정자군 (56%, 35%)과 유사한 결과를 나타냈다.
3. 또한, 정소상체 미부정자군에서 난자와 정자의 비율 (1:6000, 1:6650, 1:7700, 1:10000)에 따라 수정률과 배발달율을 조사하였던 바, 난자당 정상정자 비율이 1:6000 (68%, 32%), 1:6650 (89%, 31%)일 때 가장 높은 수정율과 배발달율을 나타내었다. 따라서, 정소상체 미부정자의 형태학적 정상정자 비율이 ≥50%일 때, 동결 융해된 사출정자와 유사한 배발달을 얻을 수 있으며, 이러한 결과로 미루어 볼 때, 정소상체 미부정자를 체외수정에 사용함에 있어서 정자의 형태학적 평가가 선행되어진다면 더욱더 효과적인 배발달을 유기할 수 있을 것으로 사료된다.

(Key words : Porcine, Epididymal /Ejaculated sperm, Embryonic development)

I. INTRODUCTION

The development of porcine embryos derived from either *in vitro* fertilization (IVF) or *in vitro* culture (IVC) reduced significantly than that of *in vivo* (Rath et al., 1995) and porcine IVF is characterized mainly by variable fertilization rates (Nagai, 1994). Until now, improving of porcine IVF system was carried out by many

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researchers and thus successful development of IVF/IVC embryos to morula and blastocyst stages was obtained (Nagai et al., 1988; Mattioli et al., 1989; Yoshida et al., 1990; Rath et al., 1995). Generally, in respect to sperm factor for IVF/IVC systems, semen quality such as volume, concentration, motility and percentage of abnormal sperm was used to predict successful IVF (Whitfield and Parkinson, 1992). However, epididymal sperm shows limited values than ejaculated sperm, because of epididymal sperm has the variation for maturity in morphology (Lacham-Kaplan and Trounson, 1994). Especially, in epididymal sperm, fertilizing ability was analyzed only through the treatment procedure such as preincubation time, concentration, culture medium (Nagai et al., 1984; Petters and Wells, 1993). On the other hand, it has known that male factor by great natural variation in shape affects to the outcome in IVF system (Kobayashi et al., 1991). Thus, the morphological evaluation of sperm in porcine IVF using epididymal sperm is important to analyze the relationship between sperm morphology and embryo development.

The objective of this study was to examine the fertilization and embryonic development rates of porcine IVM oocytes according to morphologically normal epididymal sperm in porcine IVF.

Ⅱ. MATERIALS AND METHODS

1. Collection of immature oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and cumulus-oocyte complexes (COCs) were recovered by aspiration from the follicles (2~6 mm in diameter), using a 18-gauge needle and a 10 ml disposable syringe. The COCs were washed three times with TLHEPES (1 mg/ml BSA, low carbonate TALP: Parrish et al., 1988). Only oocytes possessing a compact cumulus cell mass were selected for this experiment.

2. *In vitro* maturation (IVM)

The oocytes were transferred to maturation medium (50 oocytes/0.5 ml) and cultured in 5% CO_2, 39℃ incubator for 42~44 hr. The maturation medium was consisted of TCM-199 (Gibco) containing 10% pig follicular fluid, 25 mM $NaHCO_3$ (Sigma), 0.2 mM pyruvate (Sigma), 10 μg/ml FSH, 10 μg/ml hCG and 25 μg/ml gentamycin (Sigma).

3. Sperm preparation and morphological analysis

Epididymal semen was collected from cauda of epididymis obtained from a local slaughterhouse. Semen (1 ml) was diluted into 5 ml Sp-TALP (Rosenkans et al., 1994) and the extender was removed by washing two times at 1,000 rpm for 5 min. Then the sperm pellet was resuspended with Sp-TALP and motile sperm was collected by swimup separation after incubation for 8 min. Frozen-thawed sperm was donated from National Livestock Research Institute and highly motile ejaculated sperm was collected by percoll separation method (Park et al., 1995). Before the insemination, recovered sperm was counted and morphological normality was assessed using makler chamber (×100). Morphological analysis of sperm was used criteria described by Mortimer (1985).

4. *In vitro* fertilization (IVF) and *in vitro* development (IVD)

After *in vitro* maturation, the oocytes were transferred to the fertilization medium. Insemination was carried out according to experimental purpose as follows: 5×10^5 cells/ml epididymal sperm was inseminated to the 50 oocyte/

0.5 ml/well of 4-well dish after assessment of morphological normality in sample (Experiment 1). Over 50% groups of morphological normality in sample of epididymal sperm were adjusted to the 100% (5×10^5 cells/ml), and thus total inseminated concentration of epididymal sperm was $0.5\sim1\times10^6$ cells/ml. Also, ejaculated sperm as control group was inseminated with concentration of 5×10^5 cells/ml (Experiment 2). The fertilization medium was consisted of fatty acid free bovine serum albumin (FAF-BSA: 6 mg/ml), 0.2 mM pyruvate, 2 μg/ml heparin, PHE stock (18.2 μM penicillamine, 1.8 μM hypotaurine, 9.1 μM epinephrine) and 25 μg/ml gentamycin. The oocytes and sperm were incubated for 24 hr at 5% CO_2 in air with saturated humidity at 39℃. Embryos were cultured in NCSU 23 contained 0.4% FAF-BSA until day 3 after IVF and then transferred into NCSU 23 supplemented with 10% FBS. Blastocysts were scored on day 7 after IVF (Fig. 1).

5. Experimental design

1) Experiment 1

The fertilization and development rates were investigated according to the morphologically normal epididymal sperm at insemination and the percentage of the morphologically normal sperm against the inseminated total sperm was divided into the three groups ($\leq$10%, 10~30% and $\geq$50%).

2) Experiment 2

The $\geq$50% groups of morphological normality of epididymal sperm were adjusted to 100% (5×10^5 cells/ml), and then their fertilization and development rates were compared with those data of frozen-thawed ejaculated sperm.

3) Experiment 3

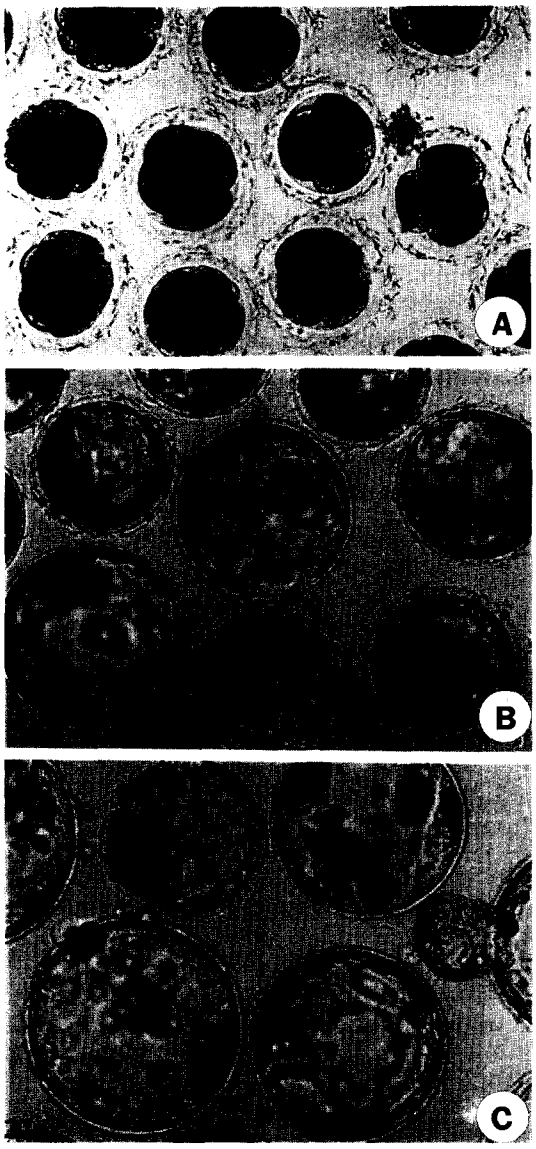

Fig. 1. Pig embryos produced by IVM/IVF/IVC (×300).
A. 3 day, B. 5 day, C. 7 day after IVF.

Based on data of experiment 2, the fertilization and development rates by using epididymal sperm were analyzed according to the oocyte: sperm ratio at insemination.

6. Statistics

A chi-square test was used to ascertain statistical differences between group. A p value < 0.01 was considered statistically significant.

Ⅲ. RESULTS AND DISCUSSION

1) Experiment 1

The fertilization and development rates were investigated according to the morphologically normal ratio of epididymal sperm at insemination (Table 1).

As shown in Table 1, the fertilization and development rates of ≥50% group (64%, 26%) were significantly higher than those of other two lower percentage groups (≤10%: 27%, 6% and 10~30%: 36%, 5%) ($p<0.01$). It is well-known that an increase in the percentage of normal sperm produced improved fertilization rates in human (Kobayashi et al., 1991). Furthermore, a high rate of fertilization *in vitro* should be led to normal subsequent embryonic development. The present results indicated that there are correlation between sperm morphology and embryonic development.

2) Experiment 2

The ≥50% groups of morphological normality of epididymal sperm were adjusted to 100% (5×10^5 cells/ml), and then their fertilization and development rates were compared with those data of frozen-thawed ejaculated sperm (Table 2).

Table 1. Fertilization and development rates of *in vitro* matured oocytes according to the morphological normality of epididymal sperm in porcine IVF

Normal sperm percentage	No. of trials	No. of oocytes examined	Cleavaged (%)	Development			
				≤MB	ExBL	≥HgBL	Total(%)
≤10	3	464	123(27)[a]	6	1	−	7(6)[a]
10~30	3	563	203(36)[a]	4	5	1	10(5)[a]
≥50	3	185	119(64)[b]	22	7	2	31(26)[b]

[a,b] Different superscripts within the same columns were significantly different ($p<0.01$).

Inseminated sperm : 5×10^5 cells/ml

MB : Middle blastocyst, ExBL : Expanded blastocyst, HgBL : Hatching blastocyst

Table 2. Comparison of *in vitro* fertilization and development rates of *in vitro* matured oocytes according to epididymal- or frozen-thawed ejaculated sperm in porcine IVF

Source of sperm	No. of trials	No. of oocytes examined	Cleavaged (%)	Development			
				≤MB	ExBL	≥HgBL	Total(%)
Epididymal*	3	278	174(63)***	28	14	3	44(27)***
Ejaculated**	3	584	328(56)***	70	34	9	113(35)***

* In group of ≥50% of normal morphology in insemination, percentage of normal sperm was adjusted to 100 (Total insem. sperm. : $0.5-1\times10^6$ cells/ml).

** Insem. sperm : 5×10^5 cells/ml.

*** Not significantly different from each other.

As shown in Table 2, the fertilization and development rates (63%, 27%) in epididymal sperm were similar to those in frozen-thawed ejaculated sperm (56%, 35%). Generally, it has been reported that the fertilization and development rates of epididymal sperm were approximately 40~60% and those of ejaculated sperm were about 21~95% in porcine IVF/IVC system (Petters and Wells, 1993) regardless of culture system condition. Especially, ejaculated sperm has a great advantage on fertilization mechanism because of repeated use of sperm with the same characteristics. In contrast, it is not possible to use epididymal sperm collected repeatedly from the same boar because of epididymal sperm are usually obtained after boars have been killed (Wang et al., 1991). However, the present results indicated that when the morphological normality of epididymal sperm in porcine IVF was more than 50, the fertilization and development rates of *in vitro* matured oocytes in porcine IVF using epididymal sperm were very similar to those of the frozen-thawed ejaculated sperm.

3) Experiment 3

Based on data of experiment 2, the fertilization and development rates using epididymal sperm were analyzed according to the oocyte: sperm ratio at insemination. In Fig. 2, the ratio of sperm per oocyte in ≥50% groups of morphologically normal epididymal sperm was represented to 1:6000, 1:6650, 1:7700 and 1:10000. Also, the fertilization and development rates were high in ratio of 1:6000 (68%, 32%), 1:6650 (89%, 31%). However, the fragmentation was increased when the ratio of sperm per oocyte was high (data not shown). This result was similar to the data reported by Xu et al. (1996), in which they indicated that the optimal ratio of sperm per oocyte was 6,250 to obtain the potentially viable embryo, although they used fresh ejaculated sperm. Therefore, this result suggested that detailed evaluation of morphological normality sperm in porcine IVF using epididymal sperm is useful in the prediction of more effective embryonic development.

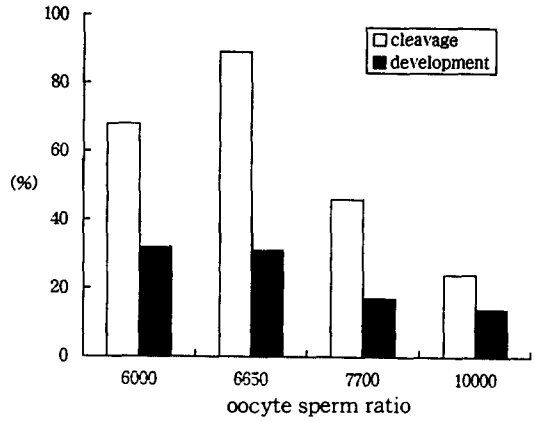

Fig. 2. Fertilization and embryonic development rate by inseminated sperm number per oocyte in porcine IVF using epididymal sperm.

Ⅳ. SUMMARY

The objective of this study was to examine the fertilization and embryonic development rates of porcine oocytes matured *in vitro* according to the morphological normality of epididymal sperm in porcine IVF. The results obtained in this experiment were summarized as follows :

1. When the ratio of morphological normality of epididymal sperm was divided into the three groups with ≤10%, 10~30% and ≥ 50%, the fertilization and embryonic development rates of ≥50% group (64%, 26%) were significantly higher than those of other two groups (≤10%: 27%, 6% and 10~30%: 36%, 5%) ($p<0.01$).

2. When the ≥50% group of morphologically normal epididymal sperm was adjusted to 100% (5×10^5 cells/ml), the fertilization and development rates (63%, 27%) of epididymal sperm were similar to those of frozen-thawed ejaculated sperm (56%, 35%).
3. Also, when the fertilization and development rates of epididymal sperm were analyzed according to the oocyte : sperm ratio (1:6000, 1:6650, 1:7700, 1:10000), the fertilization and development rates indicated high, in 1:6000 (68%, 32%) and 1:6650 (89%, 31%), the ratio of oocyte : sperm.

Therefore, when the percentage of morphological normality of epididymal sperm is more than 50, the embryonic development can be obtained similar to that of frozen-thawed ejaculated sperm. Also, these results suggested that the morphological evaluation of normal sperm in porcine IVF using epididymal sperm should be prerequisite for the more effective embryonic development.

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