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Differences in Polyspermy and Penetration Rate Following *In Vitro* Fertilization 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 체외수정후 정자침입율과 다정자침입의 차이에 미치는 영향

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요 약

본 연구는 돼지 난포란의 체외수정시 정소상체 미부정자의 형태학적 정상정자 비율에 따른 수정능의 차이를 조사하기 위하여 실시하였다. 그 결과는 다음과 같다.

1. 정소상체 미부로부터 회수된 전체의 정자중 형태학적 정상정자의 비율에 따른 (≤10%, 10~30%, ≥50%) 정자침입율과 다정자침입, 전핵형성율 그리고 난자에 침입한 평균 정자수를 조사하였던 바, ≥50%의 정자침입율과 다정자침입은 82.4%와 87.4%로 ≤10% (29.7%, 22.6%)와 10~30% (20.3%, 37.0%)보다 유의하게 높게 나타났다 ($p<0.01$). 또한, 공시된 난자의 전핵형성율도 50% 이상의 형태학적 정상정자를 가진 실험군에서 유의하게 높게 나타났다 ($p<0.01$).
2. 50% 이상의 정상정자를 가진 정소상체 미부정자를 100% (5×10^5 cells/ml)로 조정하여 수정시킨 후 그 결과를 동결 융해된 사출정자와 비교하였던 바, 다정자침입과 전핵형성율은 정소상체 미부정자 (86.7%, 35.1%)와 동결 융해된 사출정자 (86.0%, 39.4%)간에 차이를 나타내지 않았으나, 정자침입율은 정소상체 미부정자가 79.7%로 동결 융해된 사출정자의 95.5%에 비해 유의하게 낮게 나타났다 ($p<0.01$).
3. 또한, 정소상체 미부정자군에서 난자와 정자의 비율 (1:6000, 1:6650, 1:7700, 1:10000)에 따라 정자침입율과 다정자침입 그리고 전핵형성의 차이를 조사하였던 바, 수정시 난자당 정자의 수가 증가할수록 정자침입율, 다정자침입 그리고 난자에 침입한 평균 정자 수는 함께 증가하는 것으로 나타났다. 그러나, 전핵형성율은 난자당 정자의 수가 1:6000과 1:6650에서 높게 나타났다.

이상의 결과는, 돼지 난포란의 체외수정시 정소상체 미부정자를 사용했을 때, 50% 이상의 형태학적 정상정자를 가진 정자를 사용하면 동결 융해된 정자와 유사한 수정율을 얻을 수 있었으며, 정자의 형태

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학적 평가는 좀더 효율적인 수정능획득을 위해 선행되어져야 한다는 것을 시사한다고 하겠다.

(Key words : Porcine, Epididymal /Frozen-thawed ejaculated sperm, Fertilizing ability)

I. INTRODUCTION

The fertilizing ability of sperm is important to obtain of normal embryo through *in vitro* fertilization (IVF). Until now, several factors such as volume, concentration, motility and percentage of abnormal were often used for the analysis of sperm fertilizing ability (Barth et al., 1992; Whitfield and Parkinson, 1992; Wierzbowski and Kareta, 1993). Also, acrosome integrity and penetration test of zona-free hamster oocytes are used as a method of sperm analysis (O'connor et al., 1981; Berger et al., 1989). In porcine, most of studies have been evaluated about ejaculated sperm than epididymal sperm. For that reason, epididymal sperm has been problem of large variation in IVF results because repeated sperm collection was difficult from the same boar (Wang et al., 1991). Also, it has known that the fertilizing capacity of epididymal sperm has been shown difference when sperm was collected from different segments (Caput, Corpus and Cauda) of the epididymal. On the other hand, it was reported that the variable results were obtained in fertilization using epididymal sperm when the sperm quality in sample was not considered (Nagai et al., 1988).

Therefore, this study was carried out to investigate the fertilizing ability following the morphologically normal sperm ratio in porcine IVF using epididymal sperm.

II. MATERIALS AND METHODS

1. Recovery of immature oocytes and *in vitro* maturation (IVM)

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in saline (35℃ to 39℃) within 1 hr. The immature oocytes were aspirated through an 18-gauge needle into a disposable 10 ml syringe from follicles with a diameter of 2 to 6 mm. Collected immature oocytes were washed three times with TL-HEPES (1 mg /ml BSA, low carbonate TALP; Parrish et al., 1988) and matured in TCM-199 medium (with Earle's salt :Gibco, USA) containing hormones (10 μg /ml p-FSH, 10 μg /ml hCG), 0.2 mM pyruvate, and 10% porcine follicular fluid for 42~44 hr at 39℃, 5% CO_2 in air.

2. Sperm preparation and IVF

Epididymal semen was collected from cauda of epididymis obtained from a local slaughterhouse. Semen (1 ml) was diluted into 5 ml Sp-TALP (Rosenkrans 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 were collected after incubation for 8 min. by swimup method. Frozen-thawed semen 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 by using makler chamber (×100).

Insemination was carried out according to experimental purpose as follows: epididymal sperm (final concentration; 5×10^5 cells /ml) was inseminated to the 50 oocytes /0.5 ml /well of 4-well dish after assessment of morphological normality in sample (Experiment 1). Over 50% group of morphological normality of epididymal sperm was adjusted to the 100% of morphologi-

cal normality in sample (5×10^5 cells/ml), and thus total inseminated concentration of epididymal sperm was $5\sim10\times10^5$ cells/ml. Also, ejaculated sperm as control group was inseminated 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, PHE stock (18.2 μM penicillamine, 1.8 μM hypotaurine, 9.1 μM epinephrine), and heparin (2 μg/ml) and 25 μg/ml gentamycin. The oocyte and sperm were incubated at 39℃, 5% CO_2 incubator for 24 hr.

3. Morphological analysis of sperm and Diff-Quick staining

Morphological analysis of sperm was used criteria described by Mortimer (1985). Also, Diff-Quick (Kukje, Japan) staining was used to evaluate sperm morphology as follows: a) normal, b) immature, c) tail defect with hairpin, and d) immature and agglutinated sperm (Fig. 1).

4. Examination of pronuclear and polyspermy

At 24 hr after insemination, adherent sperm and cumulus mass were removed from the zona pellucida by pipetting, zona pellucida was removed by incubation with 0.05% protease for 1 min. Zona removed oocytes were fixed in 2% formalin solution for 5 min, placed on a slide and then mounted using 2.5 mg/ml Hoechst 33342 DNA label (Sigma, USA). Results were analysed as follows: oocytes were defined as penetrated when they had swollen or unswollen sperm head(s) or male proncleus(ei) in the cytoplasm. Oocytes with ≥2 pronuclei and even without a sperm tail, were also considered as penetrated. Oocytes with more than one sperm head or male pronucleus (MPN) were evaluated to be polyspermy (Fig. 2). Also, the number of sperm penetrated per oocyte was recorded.

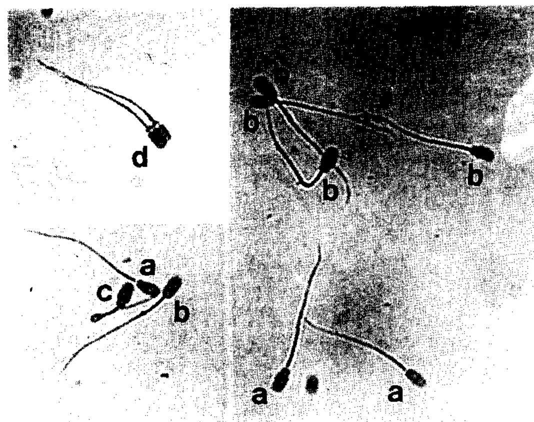

Fig. 1. Photomicrographs of Diff-Quick stained normal (a) and abnormal (b-d) porcine sperm (×300); b. immature, c. tail defects with hairpin. Inserted photograph, d. immature and agglutinated sperm.

5. Experimental design

1) Experiment 1

The penetration rate (PR), polyspermy rate (PSR), 2 pronuclei formation (2PNF), and mean number of sperm (MNS) per oocyte were evaluated according to the percentage of morphologically normal epididymal sperm at insemination (≤10%, 10~30% and ≥50%).

2) Experiment 2

The ≥50% group of morphological normality in epididymal sperm was adjusted to 100% (5×10^5 cells/ml), and then their PR, PSR, 2PNF and MNS were compared with those data of frozen-thawed ejaculated sperm.

3) Experiment 3

In experiment 2, the PR, PSR, 2PNF and MNS of epididymal sperm were evaluated according to the oocyte:sperm ratio at insemi-

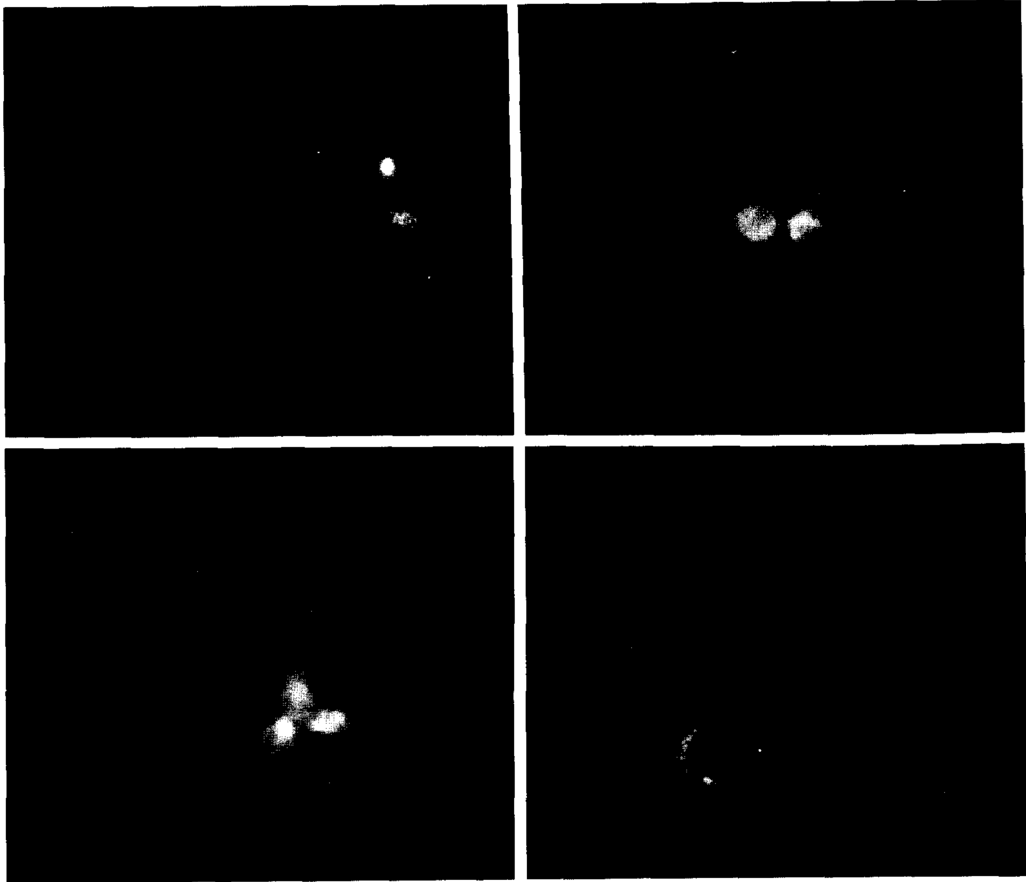

Fig. 2. Hoechst stained pig eggs (24h after IVF) (×400).
A: Metaphase Ⅱ, B: 2PN, C: Polyspermy, D: 2-cell

nation.

6. Statistics

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

Ⅲ. RESULTS AND DISCUSSION

1) Experiment 1

The PR, PSR, 2PNF and MNS per oocyte were evaluated according to the percentage of morphologically normal epididymal sperm at insemination (≤10%, 10~30% and ≥50%). As shown in Table 1, the PR and PSR of ≥50% group (82.4, 87.4%) were significantly higher than those of other two groups (≤10%; 29.7%, 22.6% and 10~30%; 20.3, 37.0%) ($p<0.01$).

The 2PNF per examined oocytes in ≥50% group (37.4%) was significantly higher than that in low percentage groups (9.1 and 5.3% for ≤10% and 10~30%, respectively) ($p<0.01$), although the 2PNF per total penetrated oocytes was not significantly different among groups

Table 1. Sperm penetration and polyspermy of *in vitro* matured oocytes according to the percentage of morphologically normal epididymal sperm at insemination

Normal sperm (%)	No. of trials	No. of oocytes examined*	No. of oocytes penetrated (%)				Mean No. of sperm / oocyte
			Total	Polyspermy	2PNF		
					A	B	
≤10	3	283	84 (29.7)[a]	19 (22.6)[a]	26 (30.7),	(9.1)[a]	1.7
10~30	3	266	54 (20.3)[a]	20 (37.0)[a]	14 (25.9),	(5.3)[a]	3.2
≥50	4	193	159 (82.4)[b]	139 (87.4)[b]	61 (38.4),	(37.4)[b]	5.2

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

* Oocytes were examined by Hoechst staining at 24 hr post insemination.

A: 2PNF per penetrated oocytes, B: 2PNF per examined oocytes.

(30.7, 25.9 and 38.4% for ≤10%, 10~30% and ≥50%, respectively). The MNS was increased as the percentage of morphologically normal sperm increases (1.7, 3.2 and 5.2 for ≤10%, 10~30% and ≥50%, respectively). In porcine, little information is available concerning the influence of sperm morphological normality on *in vitro* fertilization rates. Martinez et al. (1993) evaluated the variability of the *in vitro* penetrating capacity between boars with normal semen characteristics. In the results, they suggested that the classical parameters accepted in assessing the viability of sperm do not predict the penetrating ability of the sperm *in vitro*. But, Xu et al. (1996) reported that progressive sperm motility was a better indicator of sperm fertilizing ability and was highly correlated to estimates of oocytes penetration rate. Consequently, these results show that there are correlations between the morphological normality in sperm and the PR, PSR and 2PNF in pig IVF using epididymal sperm.

2) Experiment 2

The ≥50% group of morphological normality in epididymal sperm were adjusted to 100% (5×10^5 cells/ml), and then their PR, PSR, 2PNF and MNS were compared with those data of frozen-thawed ejaculated sperm (Table 2).

As shown in Table 2, the PSR and 2PNF were not different between epididymal sperm (86.7, 35.1%) and frozen-thawed ejaculated sperm (86.0, 39.4%), although the PR in epididymal sperm (79.7%) was significantly lower than that in frozen-thawed ejaculated sperm (95.5%) ($p<0.01$).

In conclusion, our results indicate that the PR, PSR, and 2PNF using epididymal sperm in porcine IVF can be affected by treating the morphological normality in sample. Also, the MNS of frozen-thawed ejaculated sperm per oocyte (4.9) was different compared to that of epididymal sperm (7.9) because the latter was adjusted ≥50% (5×10^5 cells/ml) to 100% ($5\sim10\times10^5$ cells/ml).

3) Experiment 3

In experiment 2, the PR, PSR, 2PNF and MNS of epididymal sperm group were evaluated according to the oocyte:sperm ratio (1:6000, 1:6650, 1:7700 and 1:10000) at insemination (Fig. 3). The PR and PSR were increased ex-

Table 2. Comparison of *in vitro* fertilization of oocytes matured *in vitro* using epididymal sperm and frozen-thawed ejaculated sperm

Source of sperm	No. of trials	No. of oocytes examined*	No. of oocytes penetrated (%)			Mean No. of sperm / oocyte
			Total	Polyspermy	2PNF	
Epididymal†	4	236	188 (79.7)[a]	163 (86.7)	66 (35.1)	7.9
Ejaculated‡	3	337	322 (95.5)[b]	277 (86.0)	127 (39.4)	4.9

* Oocytes were examined by Hoechst staining at 24 hr post insemination.

† Sperm concentration was $5\sim10\times10^5$ cells / ml.

Adjusted normal epididymal sperm (100%: 5×10^5 cells / ml).

‡ Sperm concentration was 5×10^5 cells / ml.

a,b Different superscripts within the column were significantly different ($p<0.01$).

cept PSR of 1:6650 as the oocyte : sperm ratio increases. Also, the MNS per oocyte was significantly affected by the ratio of oocyte : sperm. A major problem in porcine IVF is the incidence of polyspermic fertilization (Yoshida et al., 1990). Rath (1992) summarized that a high correlation exists between polyspermy rate and the absolute number of sperm and oocytes, but not between polyspermy rate and sperm concentration. However, this result indicated that the 2PNF was obtained highly in the low oocyte : sperm ratio (1:6000 and 1:6650) although there is not significantly different among groups. Thus, the ratio of oocyte : sperm could be critical for evaluating semen quality in IVM / IVF system.

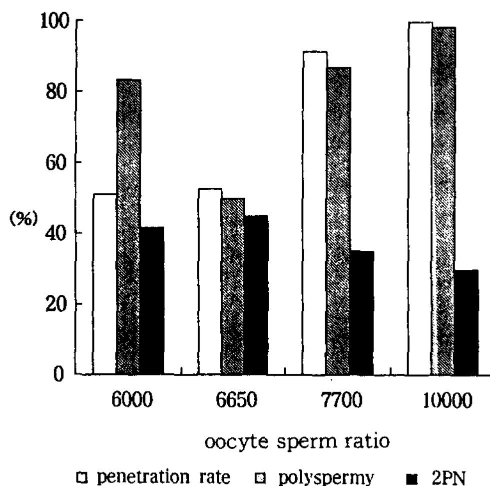

Fig. 3. Difference of *in vitro* fertility according to oocyte : sperm ratio in porcine IVF using epididymal sperm.

Therefore, these results suggested that when the percentage of morphologically normal epididymal sperm was more than 50, the fertilizing ability was very similar to that of frozen-thawed ejaculated sperm and that the detailed evaluation of morphological normality in porcine IVF using epididymal sperm should be prerequisite to obtain the more effective fertilizing ability.

Ⅳ. SUMMARY

The objective of this study was to evaluate the variation of fertilizing ability following the morphologically normal sperm ratio in porcine IVF using epididymal sperm. The results obtained in this experiment were summarized as follows:

1. When the penetration rate (PR), polyspermy rate (PSR), 2 pronuclei formation (2PNF) and mean number of sperm (MNS)

per oocyte were evaluated according to the percentage of morphologically normal epididymal sperm at insemination (≤10%, 10~30% and ≥50%), the PR and PSR of ≥50% group (82.4, 87.4%) were significantly higher than those of other two groups (≤10%; 29.7%, 22.6% and 10~30%; 20.3, 37.0%) ($p<0.01$). Also, the 2PNF per examined oocytes was significantly high in ≥50% group ($p<0.01$).

2. When the ≥50% group in epididymal sperm was adjusted to 100% (5×10^5 cells/ml), the PSR and 2PNF were not different between epididymal sperm (86.7, 35.1%) and frozen-thawed ejaculated sperm (86.0, 39.4%) although the PR in epididymal sperm (79.7%) was significantly lower than that in frozen-thawed ejaculated sperm (95.5%) ($p<0.01$).
3. Also, when the PR, PSR, 2PNF and MNS of epididymal sperm were evaluated according to the oocyte:sperm ratio (1:6000, 1:6650, 1:7700 and 1:10000) at insemination, the PR, PSR and MNS were increased as the oocyte:sperm ratio increases. However, this result indicated that the 2PNF was high in the oocyte:sperm ratio (1:6000 and 1:6650).

Therefore, these results suggested that when the percentage of morphologically normal epididymal sperm was more than 50, the fertilizing ability was very similar to that of frozen-thawed ejaculated sperm and that the detailed evaluation of morphological normality in porcine IVF using epididymal sperm should be prerequisite to obtain the more effective fertilizing ability.

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