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Effect of PN Grade on Subsequent Development *In Vitro* of Human Embryos

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사람에서 전핵 등급이 체외수정란의 발달에 미치는 영향

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SUMMARY

본 연구는 사람의 시험관아기 프로그램에서 수정 후 1일째의 전핵 등급이 체외 수정란의 발달에 미치는 영향을 조사하였다. 정상적인 시험관 아기 시술을 시행한 실례 환자를 대상으로 과배란을 유기하여 배란 직전의 난자를 채취하여 정자를 주입한 다음 체외 수정을 유도하였다. 수정 유도 18시간 후 전핵과 핵인의 형태에 따라 전핵의 등급을 1 및 2등급으로 나누어 각각 3일 동안 체외 배양을 실시하여, 체외 수정란의 형태에 따라 1, 2 및 3등급으로 분류하였든 바, 전핵의 등급에 따라 각각 1등급 체외수정란의 발달율은 1등급 전핵란에서는 83.5%로서 2등급 전핵란의 5.5%보다 유의적으로 ($P<0.05$) 높았다. 1등급 및 2등급 전핵란에서 수정 후 3일째에 5-세포기 이상의 단계로 발달하는 경우는 각각 85.1% 및 24.5%를 나타내었고, 평균 할구수는 7.4±2.1 및 4.1±3.5개를 나타내어 발달 능력에서 1등급 전핵란이 2등급 전핵란보다 유의적으로 ($P<0.05$) 높게 나타났다.

(Key words : human, embryo, *in vitro* culture, PN, follicular fluid)

INTRODUCTION

In human, the quality of the embryo and their potential for implantation are of considerable importance. Many studies have shown this relationship to justify the need for determining not only the influence for pregnancy but also more accurate assessment methods so that the maximum embryo contribution to pregnancy is achieved with the lowest possible risk of multiple conceptions.

The development of technological advances such as micromanipulation and a wealth of experience in embryo culturing techniques have resulted in an increase in embryo implantation potential. Numerous criteria have been suggested to optimize the selection process. These include the rate of embryo development, blastocyst development, ovarian/follicular vascularity, noninvasive assessment of metabolic products of embryos during development, preimplantation genetic diagnosis and morphological assessment. However, two gross measures of embryo quality which are generally employed are

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embryo cleavage and development rate, and gross morphological features, that correlate with the pregnancy rate. It has been suggested that an increase in pregnancy rate, with an increase in the number of embryos transferred, reflects only the transfer of more top grade (Hill *et al.*, 1989). 4 grades were scored by Desai *et al.* (2000) from 1 (poor morphology with fragmentation) to 4 (optimum). Puissant *et al.* (1987) presented a score whereby perfect symmetry and lack of fragmentation denoted a high morphological score, combined with a value for growth rate to give a 'vitality' score (1~6 per embryo, embryo score=cell number × fragmentation value). Steer *et al.* (1992) showed that the grade of each embryo transferred was evaluated by the number of blastomeres to produce a score for each embryo.

Furthermore recent evidence suggested that embryo grade was connected with pronuclei (PN) formation. Garello *et al.* (1999) suggested that the orientation of the PN relative to the polar bodies relates to the subsequent morphological grade of the embryo. In addition, Scott (2003) reported that assessment of PN morphology has shown correlations with implantation and development to the blastocyst. PN scoring playes a pivol role used in association with embryo development and morphology (Tesarik *et al.*, 2000; Luwig *et al.*, 2000; Wittemer *et al.*, 2000). Nucleoli are the sites where pre-rRNA is synthesized. Scoring systems based on the alignment of nucleoli has been related to the ability to continue development to the blastocyst stage (Scott *et al.*, 2000).

In these studies, PN grade in the fertilized oocytes were evaluated on the subsequent development *in vitro* grade and *in vitro* developmental potential.

MATERIALS AND METHODS

1. Patients

Twenty one cycles performed in routine IVF at the infertility clinic of Gaya Mother-Child's Hospital were analyzed. Patients were treated for all accepted indications for IVF, including male factor (n=9), tubal factor (n=7), ovulatory factor (n=3), endometriosis (n=2) and unexplained factor (n=8). Mean ages of the females were 33.6±3.4 years (range, 29~40 years). Clinical outcomes were divided into the attempt number of each cycle for each patient.

2. Controlled Ovarian Stimulation

To induce multifollicular development, controlled ovarian stimulation treatment was performed identically in all patients by a long desensitizing protocol. The protocol for ovarian stimulation was initiated with pituitary desensitization by administration of 1 mg GnRH agonist (GnRHa, D-Trp6-LHRH microcapsule, Decapeptyl, Ferring, Sweden) s.c. per day, starting in the luteal phase of the previous cycle. Serum estradiol concentrations (<50 pg/ml) and negative findings (absence of ovarian cysts > 10 mm diameter) under vaginal ultrasound scans were used to define ovarian quiescence. To induce follicular development, 150 IU of human menopausal gonadotrophin (hMG, Metrodin HP, Serono, Switzerland) was injected i.m. In case of the presence of at least three mature follicles at sonography with a diameter of 18 × 18 mm, human chorionic gonadotrophin (hCG, Profassi, Serono, Switzerland) 10,000 IU i.m. was given exactly 36 hs before oocytes retrieval.

3. Oocyte Retrieval

At 36 hrs post-hCG injection, oocyte retrieval just prior to ovulation was performed intravaginally under ultrasound guidance with an aspiration needle (Cook, France). Cumulus-oocyte complexs (COCs) were collected in 60 mm cell culture dishes (Corning, USA) under a stereo dissecting microscope (Olympus CK-2, Olympus, Japan) with trans-

mitted illumination base and heated microscope stage (MP300DM, Kitazato, Japan). After collection of COCs, its maturity stages by condition of the surrounding corona and cumulus cells were assessed. Hepes-buffered human tubal fluid (h-hTF) containing 21 mM hepes and 5% synthetic serum substitute (SSS, Irvine Scientific, USA) was used for COC washing. After washing, COCs were incubated for further maturation. in 2 ml of at 39℃ in humidified atmosphere of 5% CO_2 in air of modified human tubal fluid (m-hTF) containing 15% SSS

4. Sperm Preparation

Semen was collected by masturbation from husband. The ejaculated fresh semen was induced to liquefaction at 37℃ for 30 min. Motile sperm were isolated from fresh and liquefied semen in a two-step Percoll density gradient. In Brief, 100% Percoll solution was mixed 10X h-hTF to from 80% Percoll solution. A 40% Percoll solution was prepared from this by addition to an equal volume of h-hTF. The gradient was formed by pipetting 2 ml of 80% Percoll solution into a 15 ml conical tube and then overlaying it with 2 ml of 40% Percoll solution. The semen were placed onto the top of the 40% layer and then centrifuged at 350 ×g for 30 min. After removal of supernatant, sperm pellet was washed with m-hTF containing 5% SSS by centrifugation at 350×g for 5 min, and resuspended in 1 ml m-hTF containing 15% SSS.

5. *In Vitro* Fertilization (IVF)

At 6 hrs post-maturation, oocytes were inseminated with motile sperm at a final concentration of 50,000 sperm/oocyte. At 16~19 hrs post-insemination, eggs were removed its cumulus cells and sperm attached in ZP by repeated pipetting with a pipette, and assessed normal fertilization in which is characterized visualization of two clear pronuclei (PN) under an inverted microscope (Nikon Diaphot 300, Nikon, Japan) equipped with a Hoffman modulation contrast system. Whereas, zygotes having one PN or > three PN were classified as abnormal fertilization. PN were scored with morphological parameters of PN and nucleolus. Zygotes that categorized as grade I were defined as having a clear cytoplasmic halo, well-defined PN borders and clearly appearing nucleolus. Whereas, grade II zygotes were defined as having a vague cytoplasmic halo, dim-defined PN borders and few disappearing nucleolus (Fig. 1).

6. *In Vitro* Culture (IVC)

Media were prepared monthly in the laboratory. m-hTF and h-hTF were reconstituted with water generated from Millipore Water System (Millipore, USA), according to the protocol by Quinn (1995)

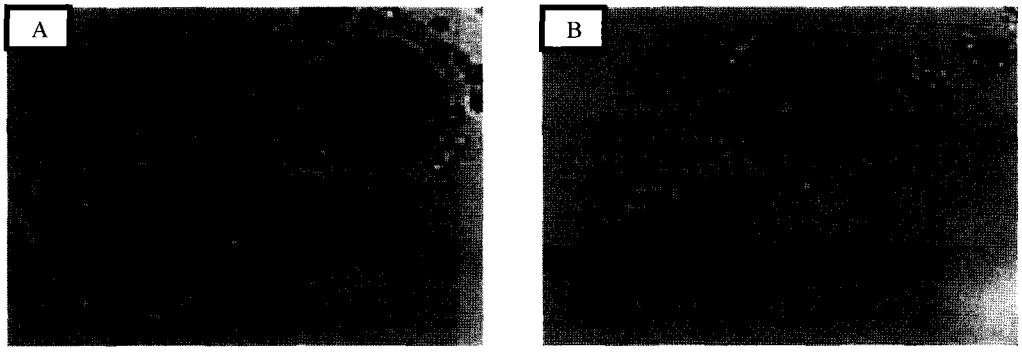

Fig. 1. Assessments of embryo grade by PN morphology at day 16~19 hrs of post-insemination (×100). A, Grade I; B, Grade II.

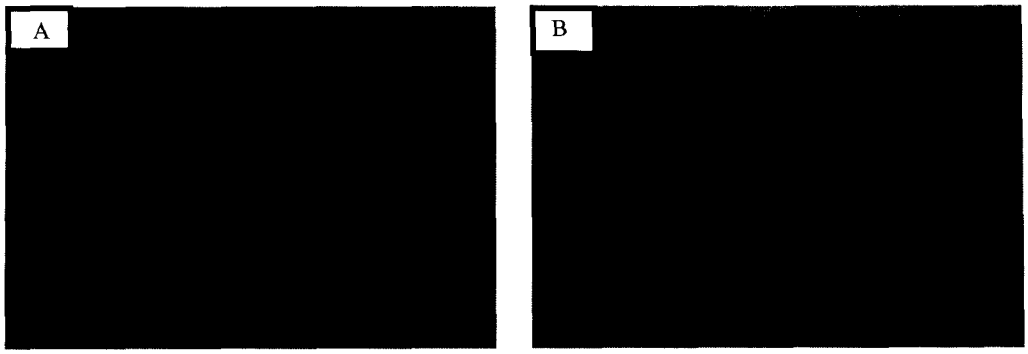

Fig. 2. Embryos from different grade evaluated by PN morphology on day 2 of post-insemination. A, Grade I; B, Grade II.

and supplemented 15% SSS. All media were screened before use with a mouse embryo bioassay. Zygotes of PN grade I and II containing two clearly visible PN were placed repetitively in the pre-equilibrated 20 μl microdrop of m-hTF containing 15% SSS (maximum 10 zygotes/drop) with cumulus cells. For further culture, every embryo was scored for the total numbers of cells, the presence of anucleate fragments and multinucleated blastomeres.

Embryo grade was defined by morphological criteria and cell numbers in the following: grade I; with no fragments, even, regular and spherical-sized blastomeres, intact zona and light and homogeneous cytoplasm, grade II; with <50% fragments, uneven-sized blastomeres and light and/or non- homogeneous cytoplasm, grade III; with > 50 % fragments, uneven- sized blastomeres and light and/or non-homogeneous cytoplasm. Grade I embryo was considered as the 'top embryo'.

7. Statistical Analysis

Differences among treatments were analyzed by SAS package program using one-way ANOVA after arc-sine transformation of the proportional data. Differences among treatments were evaluated by the chi-square test for the proportional data of the rate of embryo development and, and by the student's t-test for the cell number of embryo. Differences were considered significant when $P < 0.05$.

RESULTS

Different PN grades at day 1 embryos were compared embryos grades, cell stage and total cell numbers on day 3 of culture. At 16~19 hours post-insemination (day 1), embryos were evaluated by visualization of two clear pronuclei (PN), and categorized as fertilized oocytes with grade I or II, as assessments of PN and nucleolus morphology (Fig. 1), and followed by additionally cultured for the assessment (Fig. 2).

The results are presented in Table 1. Of oocytes that were categorized as PN grade I, all of them belonged to embryos grades I and II. Most oocytes (83.5%, 101/121) appeared to be embryos grade I, and 16.5% (20/121) revealed embryos grade II. However, in the oocytes that were categorized PN grade II, only few oocytes (6/110, 5.5%) appeared to embryo grade I. The rate of embryos grade I in PN grade I was significantly ($P<0.05$) higher than that in PN grade II (83.5% vs. 5.5%, respectively).

Following additional culture till on day 3, after categorization as grade I and II by PN and nucleolus morphology, the effects of PN grade in fertilized oocytes were compared on subsequent *in vitro* developmental stage and total cell numbers of

Table 1. Effect of PN grade on subsequent grade of the embryos*

PN grade	No. of embryos used	No. (%) of embryos in grade		
		I	II	III
Grade I	121	101 (83.5)[a]	20 (16.5)[a]	-
Grade II	110	6 (5.5)[b]	59 (53.6)[b]	45 (40.9)

[a] The grades of PN and embryos were categorized on day 1 and 3 post-insemination, respectively.

[b] Different superscripts in the same column indicate significant difference ($P<0.05$).

Table 2. Effect of PN grade on subsequent *in vitro* development and cell number on day 3 post-insemination

PN grade	No. of embryos used	No. (%) of embryos developed to			No. (mean±SEM) of cells
		1~2 cell	3~4 cell	5~8 cell	
Grade I	121	-	18 (14.9)[a]	103 (85.1)[a]	7.4±2.1[a]
Grade II	110	5 (4.5)	78 (71.0)[b]	27 (24.5)[b]	4.1±3.5[b]

[a] The grades of PN and embryos were categorized on day 1 and 3 post-insemination, respectively.

[b] Different superscripts in the same column indicate significant difference ($P<0.05$).

the embryos, as shown in Table 2. Of oocytes that were categorized as PN grade I, most of them (85%, 103/121) developed to 5~8 cell stage, and ~15% (18/121) remained at 3~4 cell stage. However, in the oocytes that were categorized PN grade II, most of them (71%, 78/110) revealed in 3~4 cell stage, and ~5% and 25% appeared at 1~2 and 5~8 cell stage, respectively. In the development to 5~8 cell stage between oocytes that were categorized as PN grade I, there were significantly ($P<0.05$) higher in grade I than that in grade II. In addition, total cell number (mean±SEM) on day 3 in the oocytes that were categorized as PN grade I were significantly ($P<0.05$) higher than that in PN grade II (7.4±2.1 vs. 4.1±3.5, respectively).

DISCUSSION

The result of this study was confirmed that the embryos of top or good grade were generated from the PN grade I zygotes and the categorization of PN grade in the fertilized zygotes can be used for the embryo prediction that have good or poor developmental potential.

Similar to the result of this study, Scott (2003) showed that the use of pronuclear oocyte morphology has shown correlations with implantation and development to the blastocyst stage in human. The pronuclear scoring, namely the presence of cytoplasmic halo, the orientation of the nuclei in relation to the polar body size, number and pattern of distribution of nucleolar precursor bodies in the nuclei were related to day 2, 3 and 5 development. Low score resulted in slow development, poor blastocyst formation and low morphology, increased fragmentation. In addition, pronuclei scoring used in association with embryo development and morphology may offer an indicator for determining which embryos would benefit from prolonged *in vitro* culture (Tesarik *et al.*, 2000;

Ludwig *et al.*, 2000; Wittemer *et al.*, 2000), particularly as the score has been related to the ability to continue development to the blastocyst stage (Scott *et al.*, 2000).

Moreover, nucleoli are visible within the respective pronuclei. The nucleoli or nuclear precursor bodies show various patterns of presentation which may suggests the chromosomal status and developmental inferiority of the embryo. Following fertilization, rRNA synthesis resumes and the nucleoli reform and grow. Thus, nucleoli allow observation of the resumption of rRNA synthesis. Zygotes with three to seven even-sized nucleoli per nucleus appear to give rise to embryos with greater developmental potential. Tesarik and Greco (1999) examined the pronuclear morphogenesis in an attempt to determine embryo viability dependent on distribution and alignment of the nucleolar precursor bodies in the respective nuclei. Embryos resulting from certain distribution patterns exhibited greater viability and rates of embryonic arrest. This group constituted the normal pronuclear stage morphology, with the remaining groups described as abnormal, attributed to asynchronous pronuclear development, which the studies believe, is harmful to embryo viability. Scott *et al.* (1998) outlined scoring systems based on the alignment of nucleoli at the junction of the two pronuclei and the appearance of the cytoplasm. These systems have been refined over time to encompass embryo morphology and development rates and include nucleoli alignment, appearance of cytoplasm and the incidence of blastomere multinucleation.

The sperm has fertilized the oocyte, the centriole and microtubules arising from the sperm bring the male and female PN into juxtaposition (Sathananthan *et al.*, 1991). Namely, the sperm-derived centriole and the microtubules arising from it are responsible for alignment of the pronuclei and syngamy. Prior to syngamy, pronuclei can be observed to rotate within the cytoplasm, directing their axes towards the second polar body (Payne *et al.*, 1997; Garello *et al.*, 1999). Thus, embryos that do not achieve an optimal pronuclear orientation may exhibit cleavage anomalies that may be observed as poor morphology, uneven cleavage of fragmentation. And preparation for mitotic division begins with two haploid pronuclei (PN) duplicating their DNA, the two PN then come together and syngamy occurs. Then, the centrioles pull the chromatids apart, a furrow appears between the two poles and the zygote cleaves into a two-cell embryo. Control of cleavage must result from control of spindle orientation, since cleavage plane orientation is dictated by the spindle. It suggested that, during the first cell cycle, these spindles consistently present small defects, problems in the formation of the mitotic apparatus may have a long-term leading to embryo mortality (Pinto-Correia *et al.*, 1993). Early entry into the first cell division has been used as an indicator of embryo viability and selection of embryos for transfer on day 2 from the cohort that undergone early cleavage by 25 hours post-insemination resulted in higher pregnancy rates (Shoukir *et al*, 1997; Sakkas *et al.*, 1998). In a study, Sakkas *et al*, (2000) postulated this increase in viability of early cleaving embryos is due to intrinsic factors regulating cleavage within the oocyte or embryo. It considered that the intrinsic factors may be including the centriole and microtubules arising from the sperm. Moreover, prior to begin preparation for first mitotic division, sperm-derived centriole must be duplicated. If centriole did not replicates or replicates more than a time by some inappropriate mechanisms and the first cleavage will be confused. For common example, tripronuclear oocytes are usually formed by dispermic fertilization and, as a result of the formation of a tripolar spindle, the majority (62%) divide into 2 cells with severely abnormal chromosome numbers and fragments (Kola *et al.*, 1987).

Martinez *et al.* (2003) investigated the rates of

pronuclear formation and the cytoskeletal organization of porcine oocytes activated by various stimuli. Oocyte activation with a protein synthesis/phosphorrylation inhibitor may have affects on distribution and function of the cytoskeleton of the oocyte different from those elicited by treatments with electric or ethanol, which result in higher proportions of oocytes with the pronucleus restrained in the peripheral ooplasm and greater oocyte fragmentation. This showed that the dynamic change in the cytoskeleton of the cytoplasm is involved during oocyte activation. For this reason, oocyte activated with various stimuli revealed that such altered microfilament architectures might be one of the causes of oocyte fragmentation. Accordingly to report of Munne and Cohen (1998), if this event such as oocyte activation fails to occur at fertilization procedure, developmental potential of embryos is limited, because sperm-derived centriole and the microfilament architectures fails to occur fully formation.

CONCLUSION

In the present study, PN grade of *in vitro* fertilized oocytes was examined and the effect of PN quality on the subsequent *in vitro* developmental potential of the embryos. At 16~19 hours post-insemination (day 1), embryos were evaluated by visualization of two clear PN, and categorized as fertilized oocytes with grade I or II, and followed by additionally cultured for the assessment of embryos grades, cell stage and total cell numbers on day 3 of culture.

From the results of the present study, the rate of embryos grade I in PN grade I was significantly ($P<0.05$) higher than that in PN grade II (83.5% vs. 5.5%, respectively). Following additional culture till on day 3, in the rate of development to 5~8 cell stage between oocytes that were categorized as PN grade I, there were significantly ($P<0.05$) higher in grade I than that in grade II (85% vs. 25%, respectively). In addition, total cell number (mean±SEM) on day 3 in the oocytes that were categorized as PN grade I were significantly ($P<0.05$) higher than that in PN grade II (7.4±2.1 vs. 4.1±3.5, respectively).

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