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Influence of Human Follicular Fluid for IVF on PN Grade and Development of Human Embryos

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사람 난포액에 의한 정자 처리가 체외수정란의 전핵과 발달에 미치는 영향

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SUMMARY

본 연구는 사람의 시험관아기 프로그램에서 체외 수정란의 질을 향상시키는 한 방법으로 난포액으로 처리된 정자를 체외 수정에 사용하여 생산된 체외 수정란의 전핵 등급과 발달 능력을 조사하였다. 정상적인 시험관 아기 시술을 시행한 실례 환자를 대상으로 과배란을 유기하여 배란 직전의 난자를 채취하여, 난포액으로 처리된 정자와 체외 수정시킨 후 사람 체외 수정란의 전핵 등급과 체외발달율을 조사하였다. 체외수정을 위한 정자의 처리 방법으로 synthetic serum substitute (SSS)를 15% 첨가한 modified human total fluid (m-hTF) 혹은 난포액에서 정자를 2시간 동안 swimming-up 처리 후 각각 체외수정에 사용한 결과, 수정율은 75.3 및 82.1%를 나타내어 유의적인 차이는 없었으나, 1등급 전핵란은 각각 48.0 및 65.5%를 나타내어 난포액에서 유의적으로 ($P<0.05$) 높았고, 배양 후 3일째에 수정란의 등급을 조사한 결과, 1등급 체외수정란은 각각 44.9 및 60.5%를 나타내어 난포액에서 유의적으로 ($P<0.05$) 높았다. 또한 체외수정란을 배양 후 3일째에 수정란의 발달 단계를 조사한 결과, 5-세포기 단계 이상을 발달하는 비율은 각각 51.0 및 70.5%를 나타내어 난포액에서 유의적으로 ($P<0.05$) 높았다.

(Key words : human, embryo, sperm, PN, follicular fluid)

INTRODUCTION

The combined IVF and ET procedure is one of many assissted reproductive technologies (ART) that have been widely used for overcoming infertility in human and animals. Although ART have evolved technically, the efficiency of IVF-ET procedures has not increased at a rate that might be expected by commitment to research in the field, judging by take-home baby rates. Many factors influence the outcome of IVF-ET. However, one of the most difficult and important aspects of IVF-ET relates to improving the embryo quality of which is most implanting after being transferred on the

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uterine wall. When the blastocyst enters the uterine cavity, it starts the complex signals that will drive embryo adhesion. Successful implantation, therefore, is determined both by the quality of the embryo and the receptiveness of the endometrium.

Commonly, mammalian ejaculated does not have ability of fertilization. To gain fertilizing ability, sperm must, either *in vivo* or *in vitro*, undergo capacitation process. Sperm washing for use in IVF-ET, which removes seminal capacitation inhibitors and prostagladins, is perhaps the common sperm preparation technique. Advanced sperm preparation techniques include the swim-up procedures, filteration Percoll gradient centrifugation, and Ficoll columns. These techniques have been used to eliminate non-motile sperm, white blood cells, and debris cells, and have been reported to increase the percentage of motile sperm. In human IVF-ET, density gradient centrifugation and swim-up procedure have been widely used as sperm preparation techniques for selecting the best sperm.

A second type of sperm preparation involves the incubation of sperm with chemical stimulant aimed at increasing sperm motility and capacitation (Lambert *et al.*, 1992). Chemical stimulants include phosphodiestrase inhibitors, pentoxifylline, and calcium transport modulators, calcium ionophore A23187. Two other techniques that stimulate sperm capaciatation are sperm refrigeration in high- albumin solutions such as TEST yolk buffer and sperm incubation with heparin (Yao *et al.*, 1999; Marin-Briggiler *et al.*, 2002).

Somewhat, a few studies showed that human follicular fluid (hFF) had been affected acrosome reaction, capacitation, attraction and motility of human sperm (Ralt *et al.*, 1991; Fabbri *et al.*, 1998; Jeon *et al.*, 2001). hFF has been frequently used as extremely useful treatment of the semen of severely dyspermic patients (Bajamonte *et al.*, 1994; Rufas *et al.*, 1998) and as second type of sperm preparation aimed at increasing sperm motility and capacitation in ART (Ralt *et al.*, 1991; Fabbri *et al.*, 1998).

The goal of this research was investigated the effects of hFF for sperm prepartion on PN grade, cleavage and developmental potential in under to enhance pregnancy rate in human IVF-ET program.

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=7), tubal factor (n=6), ovulatory factor (n=2), endometriosis (n=1) and unexplained factor (n=5). Mean ages of the females were 34.3±4.4 years (range, 29~44 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 preformed 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 hours 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 transmitted 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 modified human tubal fluid (m-hTF) containing 15% SSS at 39℃ in a humidified atmosphere of 5% CO_2 in air.

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.

To compare the rates of cleavage and development after insemination with sperm treated with or without hFF, sperm pellet collected from Perocll gradient was divided into two parts. A part of sperm pellet were suspended in 1 ml of m-hTF containing 15% SSS, the other suspended in 1 ml of hFF only. After incubation, a suspension containing motile sperm was used for standard IVF.

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.

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) 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 ㎕ 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. Preparation of hFF

Human follicular fluid (hFF) was collected by ultrasonically transvaginal-guided follicle aspiration from ovarian stimulated women participating in an IVF-ET program. After centrifugation of the hFF at 2,000 ×g for 30 min, its supernatant was taken and inactivated at 56℃ for 30 min. Inactivated hFF were filtered through 0.22 ㎛ syringe filter (Millipore, USA), and kept in frozen at −20℃ until used.

8. Statistical Analysis

Differences among treatments were analyzed bySAS 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

For this study, the effect of sperm treatments was evaluated on PN grade, embryo grade and subsequent development of embryos. The oocytes were divided into two groups for fertilization with sperm treated with swim-up either in m-hTF containing 15% SSS or inactivated hFF only for 2 hrs. The fertilization rates in m-hTF and hFF were 75.3% (98/130) and 82.1% (119/145), respectively, and did not differ (Table 1). However, the rate of grade I PN in hFF resulted in significantly ($P<0.05$) higher than in m-hTF (65.5% vs. 48.0%, respectively).

Similarly to PN grade, hFF treatment with sperm enhanced embryos grade, as presented in Table 2. The rate of grade I embryos in hFF were significantly ($P<0.05$) higher than in m-hTF (60.5 % vs. 44.9%, respectively).

Embryos fertilized with sperm either in m-hTF or hFF were compared their developmental stage on day 3 after fertilization, as presented in Table 3. In hFF, significantly ($P<0.05$) higher appearances of 5~8 cell stage embryos occurred than in m-hTF (70.5% vs. 51.0%, respectively).

Table 1. Effect of sperm treatments on PN grade in human IVF embryos

Sperm treatment*	No. of oocytes used	No. of oocytes fertilized	PN grade (%)**	
			I	II
m-hTF	130	98 (75.3)[a]	47 (48.0)[a]	51 (52.0)[a]
hFF	145	119 (82.1)[a]	78 (65.5)[b]	41 (34.5)[b]

* hFF, cultured in inactivated hFF; m-hTF, cultured in m-hTF containing 15% SSS.

** PN grade were categorized at day 1 post-insemination.

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

Table 2. Effect of sperm treatments on embryo grade categorization at day 3 post-insemination in IVF embryos

Sperm treatment*	No. of embryos used	Embryo grade (%)		
		I	II	III
m-hTF	98	44 (44.9)[a]	35 (35.7)[a]	19 (19.4)
hFF	119	72 (60.5)[b]	31 (26.1)[b]	16 (13.4)

* hFF, cultured in inactivated hFF; m-hTF, cultured in m-hTF containing 15% SSS.
[ab] Different superscripts in the same column indicate significant difference ($P<0.05$).

Table 3. Effect of sperm treatments on subsequent development at day 3 post-insemination in IVF embryos

Sperm treatment*	No. of embryos used	No. (%) of embryos developed to		
		1~2 cell	3~4 cell	5~8 cell & Mor
m-hTF	98	3 (3.1)	45 (45.9)[a]	50 (51.0)[a]
hFF	119	2 (1.7)	33 (27.8)[b]	84 (70.5)[b]

* hFF, cultured in inactivated hFF; m-hTF, cultured in m-hTF containing 15% SSS.
[ab] Different superscripts in the same column indicate significant difference ($P<0.05$).

DISCUSSION

The collected oocytes were randomly divided into two groups for fertilization with sperm treated with swim-up either in m-hTF containing 15% SSS or inactivated hFF only for 2 hrs. In this study, the effect of sperm treatments was evaluated on PN grade, embryo grade and subsequent development of embryos. Although fertilization rate showed not significantly different between sperm treatments, pretreatment of sperm with hFF in the IVF plays a pivotal role in enhancing PN grade I, embryo grade and subsequent development of embryos, and were similar to previous studies (Hourani *et al.*, 1995; Wittemer *et al.*, 1996). In a study of hamster, Lambert et al. (1992) showed that incubation of sperm with hFF provides a means of enhancing sperm penetration rate into oocytes.

The requirement for extracellular ions, such as Ca^{2+}, reflects a need for changes in the intracellular concentration of these ions during the acquisition of fertilizing ability. Namely, extracellular Ca^{2+} is required for both complete capacitation and the acrosome reaction. Sperm have revealed that modest and gradual rise in $[Ca^{2+}]_i$ occur during capacitation, and the acrosome reaction involves a rapid and large influx of Ca^{2+} (Brucker and Lipford, 1995). The rise in $[Ca^{2+}]_i$ during the acrosome reaction was due to Ca^{2+} influx via Ca^{2+} channels (Florman *et al.*, 1992; O'Toole *et al.*, 1996). Using a fluorescent Ca^{2+} indicator such as Fura-2/AM, the intercellular free Ca^{2+} concentration of individual sperm can be measured. The intercellular free Ca^{2+} concentration was increased in the single human motile sperm treated with hFF (Minami *et al.*, 1995) and hFF stimulated an increase of the intercellular free Ca^{2+} concentration of individual sperm (Shiomi *et al.*, 1996). Especially, progesterone, a component of hFF produced by granulosa and cumulus cells, has shown to stimulate a rise in $[Ca^{2+}]_i$ in human sperm (Parinaud *et al.*, 1994). Thus, the progesterone present in hFF initiated a rapid influx of extracellular Ca^{2+} into the sperm.

Oocyte activation following fertilization is ini-

tiated by sperm-induced changes in intercellular Ca^{2+} concentration. Then, it considers that the sperm treated with hFF can be enhanced oocyte activation because increase of the intercellular free Ca^{2+} concentration of individual sperm such as sham injection of high extracellular Ca^{2+} concentration from the media to activate oocyte. Some soluble sperm protein and receptor correlated with Ca^{2+} oscillation-inducing activity in oocytes has been identified. Oscillin exhibits a specific intracellular localization at the equatorial segments of the sperm head, and appropriate region for oocyte activation during fusion (Parrington *et al.*, 1996). Other factor seems to be associated with the nucleus of the sperm, especially the perinuclear theca (Kimura *et al.*, 1998). Round-headed sperm with incomplete assembly of the perinuclear materials are unable to activate oocytes when injected (Battaglia *et al.*, 1997). In addition, recognition between sperm and oocyte receptors such as hormone action in somatic cells is sufficient to induce activation. The sperm may use a G-protein coupled receptor or a tyrosine kinase receptor, etc. Although the receptor itself has not yet been identified, if sperm receptor(s) may remain a specific inner-membrane of acrosome reacted sperm. It predicts that acrosome-reacted sperm having receptor(s) as well as oscillin following fertilization may be expressed better oocyte activation. Thus, fully acrosome-reacted sperm may be induced to zygote with better PN grade. The overall mechanism through which a sperm activates an oocyte still remains largely unknown and highly controversial. Moreover, processes of sperm capacitation in the uterine or Fallopian tube remain unclear.

However, treating sperm with hFF, despite report of enhancement of sperm capacitation, motility and acrosome reaction, Franco Junior *et al.*, (1994) was comparing the rate of embryos cleavage after oocyte insemination with sperm treated in the with or without of follicular fluid, but the absence of significant differences in cleavage rate between the two experiments indicates that the quality of sperm is not changed by the addition of follicular fluid. Choi *et al.*, (1998) evaluated the effect of bovine follicular fluid (bFF) on the fertilizability and developmental capacity of bovine oocytes. Also bFF addition to fertilization medium not improved fertilizability and developmental capacity and high concentrations of bFF reduced the rate of fertilization and development.

The beneficial effects of hFF on sperm capacitation/acrosome reaction/motility have been ascribed to various follicular fluid components (Ralt *et al.*, 1991; Fabbri *et al.*, 1998; Jeon *et al.*, 2001). The most finding was the maintenance of a highly motile sperm after incubation in the culture medium added follicular fluid or hFF alone. In contradiction with, this observation was some studies in which an impairment of sperm motility in the presence of fluid (Stock *et al.*, 1989). This source of inconsistencies is precisely not known. It may be that the fluid from some follicles is indeed toxic for sperm and embryos, or deficiency of sperm activating and embryo development promoting factor(s).

The hemizona binding assay (HBA) has been developed to evaluate the tight binding of sperm to the zona pellucida, a critical event leading to fertilization. High numbers of tightly bound sperm in the assay are associated with an increased success rate in IVF (Burkman *et al.*, 1988; Coddington *et al.*, 1994). But, infertile women's sera remained sperm-immobilizing antibodies on the HBA and its have been caused low fertilization rates and poor embryo quality (Shibahara *et al.*, 2003; Chiu and Chamley, 2002). There were at least two kinds of sperm-immobilizing antibodies, one with both activities of sperm immobilization and blocking of sperm-zona tight binding and another with the former activity alone. Yao *et al.*

(1996) also shown that hFF from infertile IVF patients inhibits the binding of human sperm to the zona pellucida *in vitro* and at least two glycoproteins seem to be responsible for the inhibitory effect of hFF. Moreover, hFF from patients with endometriosis contains stronger sperm-zona binding inhibitory activity than those from patients with tubal factors, and probably those with male factors (Qiao *et al.*, 1998). This was that the deleterious substances were transported into hFF from the body fluid into tubal and endometrial environment, and affected on the sperm functions and fertilization. Preovulatory granulosa cells from endometriosis were associated with a reduced steroidogenic activity (Harlow *et al.*, 1996). The disease increases the sperm-zona binding inhibitory activity of hFF and may contribute to infertility through impairment of gamete interaction *in vivo*. In the other some studies, porcine FF from developmentally mature follicles stimulated sperm, whereas porcine FF from immature follicles inhibited sperm activity (Siegel and Graczykowski, 1991). The HBA inhibitory activity of hFF may modulate the progesterone effect and prevent polyspermic fertilization (Qiao *et al.*, 1998). However, although HBA was enhanced by follicular fluid, difference in hormonal composition, especially progesterone, was not greatly affected to HBA (Huyser *et al.*, 1997). Whatever sperm-immobilizing antibodies are known, the manipulation on gametes and embryos from patients having sperm immobilizing antibodies should be carefully carried out especially to avoid contaminating patient's serum and follicular fluid in the culture or handling medium.

Since 1999, Percoll has not been permitted for the separation of human sperm for therapeutic use because of the detrimental action of the PVP that it contains (WHO, 1999). Giorlandino *et al.*, (1998) examined that sperm with chromosomal abnormalities may results in a high rate of current abortion and semen samples from two men whose wives had histories of multiple abortions were analyzed by whole and Percoll method. Aneuploidy rates in Percoll-processed samples were higher than those found in whole specimens and aneuploid sperm also displayed greater motility. Strehler *et al.* (1998) reported that PVP has an adverse effect on the plasma, acrosomal and mitochondrial membranes of sperm. Furthermore, silica is a known irritant, and may induce an inflammatory response during IUI.

CONCLUSION

The goal of this research is to improve the embryo quality for enhancing pregnancy rate in human IVF-ET program. Although multiple factors influencing the pregnancy rate are in existence, one of the most complicated but important subjects of ART may be the improvement in embryo quality. In the present study, the PN grade, cleavage rate, and *in vitro* developmental potential of the IVF embryos with sperm treated with hFF were examined comparatively.

From the results of the present study it was revealed that human oocytes collected were fertilized *in vitro* with sperm treated with swimming-up in m-hTF supplemented with SSS or inactivated hFF for 2 hrs. Though the fertilization rate was not significantly different between sperm treatments, the rate of grade I PN in hFF resulted in significantly ($P<0.05$) higher than in m-hTF (65.5% vs. 48.0%, respectively). The rate of grade I embryos in hFF were significantly ($P<0.05$) higher than in m-hTF (60.5% vs. 44.9%, respectively). Moreover, embryos fertilized with sperm either in m-hTF or hFF were compared their developmental stage on day 3 after fertilization, in hFF, significantly ($P<0.05$) higher appearances of 5~8 cell stage embryos occurred than in m-hTF (70.5% vs. 51.0%, respectively).

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