

EFFECTS OF HUMAN FOLLICULAR FLUID AND SYNTHETIC SERUM SUBSTITUTE ON HUMAN EMBRYONIC DEVELOPMENT AND CELL CLEAVAGE

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Abstract– The development of culture media able to mimic the preovulatory stage of follicular environment and support nuclear and cytoplasmic maturation of oocyte is important for in-vitro fertilization (IVF) programs. It seems that the best culture media for embryonic development and cell cleavage is the natural composition which surrounds the oocyte which has been used occasionally in human IVF programs. For further investigation of effects of natural media composition of human follicular fluid (HFF) on embryo development, we compared the biochemical constituents of HFF with synthetic serum substitute (3S) and their effects on embryo development and cell cleavage. From a total of 40 women with unexplained infertility, who attended for intracytoplasmic sperm injection (ICSI) in IVF center of Mirzakoochak Khan Hospital, we collected the HFF during oocyte pick-up in operation room. The chemical composition of HFF was compared to 3S medium culture to identify which natural components of follicular fluid might enhance embryo maturation *in vitro*. The results of comparison between HFF and 3S culture media indicated significant differences in biochemical component except for Na and bilirubin concentration and pH level ($P<0.05$) and significant differences between the rates of cell cleavage in 3S compared to HFF media ($P<0.05$). Furthermore the rate of embryo cell cleavage related to HFF is faster than 3S medium. There was no significant difference between the development of embryos in 3S and HFF media culture. Our data confirm the benefit of the use of HFF as a culture medium.

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INTRODUCTION

Since the birth of the first baby tube achieved through conception outside the human body in 1978,

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the principles of “*in vitro*” (literally “in glass”) fertilization and culture have remained the same. Careful establishment and maintenance of a well-controlled, sterile environment in which the normal physiology of fertilization and early development can be played out to provide healthy embryos for transfer back into the uterus have remained relatively undisturbed. However, during the last two decades great advances have been made in the development of more appropriate culture media, which have enabled embryos to be grown for extended periods of

HFF and human embryonic development

time in culture, and decreased cost of cultures. The development of new culture media enables to mimic the preovulatory stage of follicular environment and supports nuclear and cytoplasmic maturation of oocyte (1, 2) in women who have low ovarian stimulation (3). Body fluids such as amniotic fluid, sera, peritoneal fluid (4) and human follicular fluid (HFF) (5) have been used in human IVF programs. HFF is a complex extracellular fluid that is semiviscous and yellow in color. It is a derivative of blood plasma and secretions synthesized in the follicle wall (6). Endocrinological changes in follicular fluid may influence steroidogenesis, oocyte maturation, and ovulation (7). Moreover HFF inhibits zona hardening, promotes hyperactivation and acrosome reaction of sperm, stimulates oocyte maturation, increases fertilization rate and enhances preimplantation embryo development. Further, it contains high levels of gonadotropins and growth factors, which could be beneficial for oocyte maturation and embryo development (8). Studies on oocyte maturation have involved the addition of follicular fluid to the maturation medium. Supplementation with follicular fluid has been demonstrated to support *in vitro* nuclear and cytoplasmic maturation (9).

The aim of this study is to investigate the effects of natural biochemical components of HFF on embryo development and cell cleavage as well as to compare it with synthetic serum substitute (3S) medium, which is an expensive synthetic media in IVF programs. The concentration of selected molecules (such as glucose and albumin), electrolytes, lipids, steroids, pH and osmolarity of follicular fluid is compared with 3S medium in order to identify any component of follicular fluid which might enhance embryo maturation *in vitro*.

MATERIALS AND METHODS

A total of 40 women with unexplained infertility, undergoing intracytoplasmic sperm injection (ICSI) in IVF center of Mirzakoochak Khan Hospital participated in the study. The age of the women ranged between 15-37 years (mean 27.37±6.14). HFF was collected from the patients who were negative

for HIV infection, syphilis and hepatitis B antigen to prevent infection. We obtained informed consent from all patients.

Follicular fluid collection, preparation and analysis

During the oocyte aspiration in the human IVF programs, HFF was collected from follicles that were >18 mm in diameter. Whenever possible non-blood-stained HFF was collected, but the fluid mixed with a small amount of blood was also collected. The fluid was centrifuged at 2000 rpm for 20 minutes and the supernatant was heat-inactivated at 56°C for 35 minutes. The heated fluid was cooled and sterilized with a filter (0.22 µm-pore-size filters). All HFF samples were kept at -4° C until the time of the culture.

Follicular fluid samples were subjected to a range of biochemical measurements. Samples were all defrosted at room temperature and analyzed after daily calibration of the machines by the analysis of appropriate controls. A total of 16 tests were carried out on follicular fluid, using Zist Chimi and Pars Azmoon kits.

Oocyte and sperm collection and preparation

Patients underwent the long protocol (gonadotrophin releasing hormone agonist) for ovarian stimulation. The cycle was started by initial suppression of ovarian function with a gonadotrophin releasing hormone (GnRH) analogue. After ultrasonographic verification of down-regulation of ovaries and endometrium, daily intramuscular injections of human menopausal gonadotrophin (HMG) were given. This was continued until the follicle size reached to a diameter of 16-18 mm and the serum concentrations of oestradiol reached 500 pgr/ml. Then a single dose of human chorionic gonadotrophin (HCG, 10000 IU) was given intramuscularly to induce follicular and oocyte maturation (10).

Oocyte pick-up was performed by aspirating follicle transvaginally, using ultrasound monitoring. Cumulus cells of cumulus-enclosed oocytes were removed by using hyaluronidase enzyme and then oocytes were incubated for 2-4 h in humidified

condition containing 5% CO₂ air at 37°C. After 2-4 h, oocyte morphology was assessed and those showing clear ooplasm and extruded first polar body metaphase II were selected for sperm injection.

Sperm cells for ICSI were prepared using the swim-up procedure in Ham's F-10 supplemented with 5% human albumin (9). ICSI was carried out basically according to the procedures by Palermo *et al.* (11) and Van Steirteghem *et al.* (12). The injected oocytes were then transformed to either 3S or HFF medium covered by lightweight paraffin oil and stored in an incubator containing 5% CO₂ air at 37°C.

Assessment of embryonic development

At 48 h after ICSI, embryonic development was assessed under the inverted microscope. Embryos were classified based on morphological criteria as described before (8):

Grade I: Embryos with blastomeres of equal size and no cytoplasmic fragments.

Grade II: Embryos with uneven-size blastomeres and no cytoplasmic fragments.

Grade III: Embryos with partial anucleate fragments (25% to 50% cytoplasmic fragments).

Grade IV: Embryos with higher fragmentation rates (50% < fragments < 75%).

Grade V: Embryos with few blastomeres of any size associated with severe or complete fragmentation (75% < fragments).

Grade VI: Degenerated embryos.

Grade I, II, III embryos were transferred, and grade IV, V, VI were excluded.

Statistical analysis

The statistical significance of the results was evaluated by the χ^2 test. The results, expressed as means \pm SEM were compared using the Student's *t* test. Differences were considered statistically significantly if *P* value was ≤ 0.05 with use of a two-tailed test.

RESULTS

Values in table 1 represent mean \pm SEM concentration of selected molecules, electrolytes, lipids, steroids, pH and osmolarity in HFF and 3S

Table 1. Concentration of biochemical constituents of human follicular fluid in normal ovaries and synthetic serum substitute

Biochemical	HFF	3S	<i>P</i> value
Na (meq/dl)	148 \pm 2.36	144	0.49
K (meq/dl)	4.03 \pm 0.06	4.28	0.0001†
P (mg/dl)	3.7 \pm 0.1	0.2	0.0001†
Ca (mg/dl)	8.47 \pm 0.22	7	0.0001†
Mg (mg/dl)	1.72 \pm 0.04	0.5	0.0001†
Fe (mg/dl)	78.44 \pm 1.92	1.9	0.0001†
Cholesterol (mg/dl)	22.89 \pm 1.04	18	0.001†
Triglyceride (mg/dl)	115.45 \pm 43.1	6.1	0.0001†
Total Protein (g/dl)	5.61 \pm 0.51	2.1	0.006†
Albumin (mg/dl)	3.74 \pm 0.05	1.4	0.0001†
Glucose (mg/dl)	62 \pm 3.16	106	0.0001†
Bilirubin (mg/dl)	0.11 \pm 0.009	0.1	0.083
Oestradiol (pg/ml)	1301.9 \pm 18.1	8	0.0001†
Testosterone (ng/dl)	13.12 \pm 1.07	0.9	0.0001†
Osmolarity (mmol)	282.57 \pm 1.02	280	0.022†
pH	7.53 \pm 0.05	7.5	0.505

Abbreviations: HFF, human follicular fluid; SSS, synthetic serum substitute.

*Data are given as mean \pm SEM.

†Significant.

culture media. Also shown are the results of statistical analysis. Significant differences were seen in all of the items except Na and bilirubin concentration and pH level (*P*<0.05). K concentration was lower and P, Ca, Mg and Fe concentrations were higher significantly in HFF relative to 3S culture medium. Of 72 embryos selected for ICSI, a total of 43 were cultured in 3S (59.7%) and a total of 29 were cultured in HFF (40.3%).

Tables 2 and 3 show the results of embryonic development and cell cleavage rate in HFF and 3S culture media after 48h of microinjection.

Table 2. Comparison of cell cleavage in HFF and 3S culture media after 48h of microinjection*

Embryos	3S (n=43)	HFF (n=29)
2-4 cell stage	37(86.1%)	19(65.5%)
8-16 cell stage	6(13.9%)	10(34.5%)

Abbreviations: HFF, human follicular fluid; 3S, synthetic serum substitute.

*Data are given as number (percent).

HFF and human embryonic development

Table 3. Comparison of embryonic development in HFF and 3S culture media after 48h of microinjection*

Embryos	3S (n=43)	HFF (29)
I	35(81.3%)	19(65.5%)
II	6(14%)	10(34.5%)
III	2(4.7%)	0 (0%)

Abbreviations: HFF, human follicular fluid; 3S, synthetic serum substitute.

*Data are given as number (percent).

37 embryos (86.1%) in 3S medium and 19 embryos (65.5%) in HFF were at 2-4 cell stage. Six embryos (13.9%) in 3S medium and 10 embryos (34.5%) in HFF were at 8-16 cell stage. Significant differences were seen between the embryonic cell cleavage in 3S and HFF media ($P<0.05$). It seems that the cell cleavage of embryos in HFF is faster than 3S medium (Table 2). There was no significant difference between the embryonic development in 3S and HFF media culture. Out of 43 embryos cultured in 3S medium, 35 were in grade I (81.3%), 6 were in grade II (14%) and two were in grade III (4.7%). Out of 29 embryos cultured in HFF medium, 19 were in grade I (65.5%) and 10 were in grade II (34.5%) (Table 3).

DISCUSSION

Since the follicular fluid is in intimate contact with the oocyte and granulosa cells, changes in the concentration of its different components may reflect the requirements of follicular structure (13). Numerous studies have been conducted in order to measure the constituents of follicular fluid and to assess the correlation of these constituents with embryonic maturation. The chemical composition of follicular fluids from various species has been well documented (14).

The study of Collins *et al.* on the biochemical composition of equine follicular fluid showed lower concentrations of all proteins and higher concentrations of lipids in follicular fluid than serum (15). On the other hand, the findings of Shalgi *et al.* showed that both the concentration of total protein and its composition were the same in HFF and serum (16). In the study of David *et al.* the concentration of sodium, chloride, glucose, and

protein of rabbit follicular fluid were not significantly different from plasma (17).

In our experiment, the chemical composition of HFF is compared with 3S, the most widely used culture medium in IVF programs in order to base embryo culture media on the environment of the female reproductive tract. But whether human embryos could be exposed to a natural environment such as HFF in order to confront the major drawbacks of synthetic environment such as high cost and undisclosed composition is not clear. To answer this question, we tried to use HFF as a culture medium and to assess the effects of its use on the embryos development and cell cleavage after ICSI. It seems that embryonic cell cleavage in FF is faster than 3S medium. Other stages are comparable in two media. Our data are in agreement with those obtained in the study of Dell'Aquila *et al.* in which the cleavage rate of the number of oocytes matured in the presence of HFF were significantly higher than those of the control oocytes (9). Concentrations of steroid hormones in the follicular fluid have been positively correlated with nuclear and cytoplasmic maturation of oocytes in stimulated women (9). Another theory for the increased cell cleavage may be the specific activities of HFF. The current data confirm and extend these observations by demonstrating that HFF is strongly mitogenic, chemotactic and angiogenic (18). We think that the faster cell cleavage in HFF medium may be the result of these activities. In contrast Choi *et al.* reported that the inhibitory action of follicular fluid on *in vitro* maturation, male pronucleus formation and developmental capacity of bovine oocytes is dependent on the developmental stage of the follicles from which fluid was obtained (19). Kim *et al.* reported that a higher-dose (60%) of follicular fluid (from follicles 1 to 5 mm diameter) added to the maturation medium inhibits development as well as oocyte maturation, probably due to coagulating the cumulus cell mass by fibrin-like substance in the follicular fluid (20). Also some results indicate that follicular fluid contains an inhibitory factor(s) originally termed the oocyte maturation inhibitor. These inhibitors seem to decrease the process of follicle development and ability to ovulate (20).

Dell'Aquila *et al.* reported that follicular fluid didn't increase the maturation of oocytes to metaphase II stage compared to TCM-199 medium on the *in vitro* fertilization (9). Yao *et al.* reported that HFF inhibit the binding of spermatozoa to zona pellucida *in vitro* (21). More detailed studies are required to evaluate the mechanism by which HFF acts on the oocyte.

In conclusion, our data confirm the benefit of the use of HFF as a culture medium. Our data indicate that HFF contains a variety of components and activities that are able to support the development of oocytes in IVF programs.

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HFF and human embryonic development

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