Autologous mature follicular fluid: its role in in vitro maturation of human cumulus-removed oocytes

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Objective: To assess the effects of autologous mature follicular fluid (FF) on the in vitro maturation (IVM) outcome of human cumulus-removed oocytes from routine stimulated ovaries, particularly on the IVM co-culture outcome with autologous cumulus cells.
Design: Prospective comparison study.
Setting: A provincial reproductive medicine and research center.
Patient(s): A total of 196 germinal vesicle (GV) and 192 metaphase I (MI) stage oocytes were recruited from 151 infertile women by ovarian stimulation for routine intracytoplasmic sperm injection (ICSI) program.
Intervention(s): Two novel IVM systems assisted with either autologous mature FF alone or both autologous mature FF and autologous cumulus cells were developed to mature in vitro MI and GV stage oocytes, respectively. A standard IVM system was used as the control.
Main Outcome Measure(s): Cumulative IVM rate of oocytes and the subsequent fertilization, cleavage, and preimplantation embryonic development.
Result(s): By adding autologous mature FF to the medium of the IVM system, the nuclear maturation of human cumulus-removed oocytes was significantly promoted in both speed and rate, but not the corresponding fertilization, cleavage, and preimplantation embryonic development.
Conclusion(s): Autologous mature FF favors the IVM of human cumulus-removed oocytes, including the IVM co-culture with autologous cumulus cells, mainly in nuclear, but not cytoplasmic, maturation. (Fertil Steril® 2008;90:1094–102. ©2008 by American Society for Reproductive Medicine.)

Key Words: In vitro maturation, oocyte, autologous follicular fluid, autologous cumulus cell, in vitro fertilization–embryo transfer

In routine IVF or intracytoplasmic sperm injection (ICSI), about 15% of oocytes collected during ovarian aspiration in an ovarian stimulation cycle were immature (1, 2). These oocytes cannot be used for ICSI because of their immature state and they are usually discarded when they are denuded from the cumulus cells (3–6).

Although some of these immature oocytes may be atretic or may have been resistant to in vivo gonadotropin stimulus, many are still capable of undergoing spontaneous maturation and fertilization if appropriate culture conditions are present in vitro (7). On this basis the in vitro maturation (IVM) technique was introduced to assist the spontaneous IVM of human oocytes as a "rescue" strategy (1, 2).

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Previous studies have shown that the IVM rate of cumulus-removed oocytes and the subsequent fertilization and development potential were all lower than those of cumulus-enclosed oocytes (6, 8). Therefore the existence of cumulus cells has a positive impact on the maturation and subsequent development of oocytes. This observation is confirmed by studies in other mammals (9–13).

In humans, attempts have been made to improve the IVM of human oocytes by IVM co-culture with helper somatic cells, including heterologous monkey kidney cells (7), homologous sperms (14) or cumulus cells (15), and autologous cumulus cells (5, 16). However, the results of IVM co-culture were not consistent, in particular, no significant improvement was obtained in the maturation rate of oocytes from IVM co-culture with autologous cumulus cells when compared with the conventional non-IVM co-culture (5, 16).

Here, IVM co-culture of oocytes with autologous cumulus cells belongs to a particular individual. It is safe from the risk of pathogen transmission and, thus, superior to those IVM co-culture systems supplemented with non-autologous components, such as heterologous animal feeder cells and homologous preovulatory follicular fluid (FF) (1, 17). Individual IVM co-culture of oocytes with autologous cumulus cells should be developed.

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Evidence is accumulating that preovulatory FF of human, the mature FF of human, contains high levels of nutrients, gonadotropins, growth factors (18), soluble antiapoptotic Fas (19), meiosis-activating sterol (20), and other unidentified components that are helpful for oocyte maturation and embryo development (21). Previous studies have reported that human mature FF, as a heterologous or homologous supplement to the culture medium, stimulates the meiotic resumption of mouse oocytes (22) and promotes the IVM of human oocytes in the form of a cumulus–oocyte complex from either unstimulated (23) or stimulated ovaries (23, 24). A similar situation occurred in studies carried out on other mammals (25–28). Human mature FF appears to be a suitable medium or supplement in the IVM culture system (22–24). However, few data are available on the effects of autologous mature FF on the IVM of human cumulus-removed oocytes.

In the present study, prospective comparison experiments were conducted to assess the effects of autologous mature FF on the IVM of human cumulus-removed oocytes, particularly on the IVM co-culture with autologous cumulus cells. We hypothesized that, during the IVM co-culture of human cumulus-removed oocytes with autologous cumulus cells, the concurrently cultured autologous cumulus cells may have not reached the feeder layer-like state at the initial period of IVM co-culture, and that this insufficient or relatively delayed impact of autologous cumulus cells on IVM co-culture may be attributed to the insignificant improvement in IVM. Adding a certain amount of autologous mature FF to the medium of the IVM system may provide not only an immediate support of autologous mature FF to IVM, but also, through stimulating effects of autologous mature FF on the growth of autologous cumulus cells, an indirect compensation for the insufficient or relatively delayed support of autologous cumulus cells to IVM at the initial period of IVM co-culture.

MATERIALS AND METHODS

This study was undertaken after complete institutional review board approval had been obtained from the Women's Hospital, University of Zhejiang. A written informed consent was also obtained from each infertile couple before the use of their donated gametes.

Source of Human Gametes

A total of 388 morphologically normal immature oocytes (196 at prophase of meiosis I [GV stage] and 192 at metaphase of meiosis I [MI stage]) were recruited from 151 consecutive infertile women (31.3 $\pm$ 3.9 years; mean $\pm$ SD) in 156 ovarian stimulation cycles for routine ICSI. Those immature oocytes without simultaneously in vivo-matured sibling were thought not appropriate study subjects and were not recruited in the present study. Obviously dysmorphic or degenerated immature oocytes were excluded. Detailed criteria for exclusion are as in the study by Ng et al. (29), that is, if an immature oocyte showed septum, extension, uneven thickness, or a nonspherical shape in its zona pellucida (ZP), or saccules, vacuoles, refractile bodies, localized granularity, dark area or irregular shape in its cytoplasm, it was regarded as dysmorphic or degenerated. The same original sperm were used in the ICSI of both autologous in-vivo matured and in-vitro matured oocytes from each infertile woman.

Ovarian Stimulation Protocols and Oocyte Retrieval

Ovarian stimulation was performed using a GnRH agonist (Decapeptyl; Ferring GmbH, Kiel, Germany) and recombinant human FSH (Gonal-F, Serono, Geneva, Switzerland) in a long or short protocol. In the present study, the majority (92.3%, 144/156) of the infertile women used the long protocol for their ovarian stimulation. The oocyte retrieval was performed 34–36 hours after hCG (Profasi, Serono) injection with ultrasound-guided needle puncture.

IVM Protocols and Preparation of Corresponding IVM Medium

Standard IVM protocol This protocol was used as a control in the present study. The commercial MediCult IVM System (MediCult Co., Jyllinge, Denmark) was used according to the manufacturer's instructions with some modifications. The IVM medium of this system was used as the basal IVM medium, in which 0.075 IU/mL recombinant human FSH, 0.1 IU/mL hCG, and 10% (v/v) serum substitute supplement (Irvine Scientific, Santa Ana, CA) were supplemented. The resultant Standard IVM working solution was equilibrated in an incubator at 37°C, 5% CO_2, and water-saturated atmosphere for at least 4 hours for the succeeding IVM.

Autologous mature FF-assisted IVM protocol (autologous mature FF IVM) This protocol was developed to assess the effects of autologous mature FF alone on the IVM of MI stage oocytes and the subsequent development of in vitro-matured oocytes in the present study. In this protocol, 10% (v/v) autologous mature FF was added to the Standard IVM working solution prepared according to the Standard IVM protocol. The 10% volume of autologous mature FF used in the present study was based on research results reported previously by Somigliana and colleagues (30), in which a significant enhanced ability to stimulate proliferation of human endometrial cell growth in vitro was demonstrated by using the same volume of human mature FF. The autologous mature FF was retrieved from the dominant follicle of each infertile woman at oocyte pickup without blood contamination. After 600 $\times$ g centrifugation for 20 minutes to remove the cellular debris and filtration of the supernatant with a 0.22-μm filter, the resultant autologous mature FF was ready to be used.

Autologous mature FF and autologous cumulus cell-assisted IVM co-culture protocol (autologous mature FF & cumulus cell coIVM) This protocol was developed to assess the effects of both autologous mature FF and autologous cumulus cells on the IVM of GV stage oocytes and the subsequent development of in vitro-matured oocytes in the

present study. In this protocol, both autologous mature FF and autologous cumulus cells were added to make the IVM co-culture working solution. The autologous mature FF was the same as used in the autologous mature FF IVM protocol. The autologous cumulus cells of the same patient were collected just after they were removed from their oocytes at the time of oocyte preparation for ICSI. These collected cumulus cells were pooled together in 1.5 mL of modified human tubal fluid (HTF) medium with gentamicin–HEPES (M-HTF; Irvine Scientific). Then the cumulus cells were gently uploaded on an equal volume of prewarmed lymphocyte separation solution (Bio Basic, Inc., Shanghai, China) in a centrifuge tube and centrifuged at 600 × *g* for 20 minutes at 18°–20°C to separate the autologous cumulus cells from the red blood cells. The purified autologous cumulus cells were aspirated at the interface, washed with three volumes of Dulbecco's phosphate-buffered saline solution (Irvine Scientific) and centrifuged at 300 × *g* for 5 minutes at 18°–20°C. The supernatant was then removed and the pellet was suspended in three volumes of equilibrated Standard IVM working solution prepared according to the Standard IVM protocol and centrifuged again at 300 × *g* for 5 minutes at 18°–20°C. The supernatant was removed again, the number of the purified autologous cumulus cells was counted with a hemacytometer, and the viability was assessed with trypan blue exclusion. Only the purified autologous cumulus cells whose viability was ≥90% were used to develop the corresponding IVM co-culture. After regulating the density of the qualified autologous cumulus cells to 2.5 × 10^5/mL with the same equilibrated Standard IVM working solution, as mentioned previously, and adding the same patient's autologous mature FF (10% [v/v]) prepared according to the autologous mature FF IVM protocol, the resultant autologous mature FF & cumulus cell coIVM working solution was ready to be used.

IVM Study Design and the Detailed IVM Culture

The maturity of each oocyte and its stage in meiosis (GV or MI stage) was confirmed using ×200 magnification on an inverted microscope. Randomized block design was used in this study. Autologous or sibling oocytes at the same meiosis stage (GV or MI) were classified to the same block and the oocytes of the same block were randomized to be allocated to different IVM protocols.

All oocytes' IVM culture of the same block were carried out in the same culture mode of microdrops (30 μL) overlaid with prewarmed embryo-tested light mineral oil (Irvine Scientific) in a standard tissue culture dish at the same incubator atmosphere (37°C, 5% CO_2, and 100% saturation humidity); only the IVM medium in the microdrops was different according to the different IVM protocols.

The total duration of IVM was 48 hours. The maturity of oocytes during the IVM was assessed with photographed evidence every 6 hours (daytime hours) or 12 hours (nighttime). The extrusion of the first polar body was the marker of oocyte maturation and indicated that the oocyte had reached metaphase II of meiosis.

As soon as oocyte maturation was confirmed, ICSI treatment and subsequent observation of fertilization and embryonic development followed.

ICSI Procedure and Embryonic Culture

The same sperm as used in the autologous in vivo-matured oocytes was used during the ICSI procedure. The swim-up technique was used for the sperm preparation in the present study according to the World Health Organization (WHO) laboratory manual (31).

A standard ICSI procedure, as described by Palermo et al. (32), was performed on each in vitro-matured oocyte as on each in vivo-matured oocyte.

After ICSI, the injected oocytes were washed in fresh HTF medium (Irvine Scientific) containing 10% (v/v) serum substitute supplement, and cultured (37°C, 5% CO_2, and 100% saturation humidity) individually in droplets of the same fresh HTF medium.

Fertilization was evaluated 16–18 hours after ICSI. Oocytes with two pronuclei and the extrusion of second polar body were considered normally fertilized. The fertilized zygotes were washed and cultured (37°C, 5% CO_2, and 100% saturation humidity) individually in freshly prepared P1 medium droplets (Irvine Scientific) containing 10% (v/v) serum substitute supplement and overlaid with embryo-tested light mineral oil.

On day 3, the embryos were transferred and cultured (37°C, 5% CO_2, and 100% saturation humidity) individually in droplets of blastocyst medium (Irvine Scientific) containing 10% (v/v) serum substitute supplement and overlaid with embryo-tested light mineral oil. Then the transfer of embryos into droplets of fresh blastocyst medium was carried out every 48 hours until day 5 after ICSI.

During the embryo culture, developmental stage and embryonic quality observations were conducted every 24 hours. In the present study, the embryo that showed normal cleavage speed (at least 8 cells at day 3 after ICSI), uniform or slightly irregular blastomeres, less than 10% of anucleate fragmentation occupying the embryo volume, and nongranular cytoplasm was considered as good quality (33, 34).

Statistical Analysis

Descriptive statistics in proportions were used to describe the general characteristics of the IVM of MI and GV stage oocytes. Chi-square and Fischer exact tests, when appropriate, were used to compare IVM outcome and subsequent development among different IVM protocols. The descriptive statistics in proportions on the ICSI fertilization of autologous in vivo-matured oocytes and the subsequent development were also used to show the difference of this aspect between in vivo-matured oocytes and in vitro-matured oocytes. All *P* values are two-sided and $P<.05$ was considered statistically significant.

RESULTS

Time Course of the Maturation of M1 and GV Oocytes Under Different IVM Protocols

Figures 1 and 2 show the cumulative time course of maturation for M1 and GV oocytes, respectively, under different IVM protocols. The cumulative maturation rates of MI stage oocytes at various durations of autologous mature FF IVM protocol were all higher than those of MI stage oocytes in the Standard IVM protocol. The differences at 24-hour IVM between the two IVM protocols for M1 oocytes were statistically significant (Fig. 1). Among the two IVM protocols used in the IVM of GV stage oocytes, the cumulative maturation rates with autologous mature FF & cumulus cell coIVM at both 36- and 48-hour IVM were all significantly higher than those with the Standard IVM protocol (Fig. 2).

Fertilization, Cleavage, and Embryonic Development of Oocytes Matured Through Different IVM Protocols

In the present study, some in vitro-matured oocytes (17/178, 9.6% from MI stage oocytes and 18/146, 12.3% from GV stage oocytes) were not subjected to ICSI because of the presence of oocyte degeneration after IVM or failure to perform ICSI within a 6-hour period after the second polar body extrusion. The detailed criteria for the presence of oocyte degeneration were the same as mentioned in the Materials and Methods section, Source of Human Gametes. Table 1 shows the fertilization, cleavage, and embryonic development of oocytes matured through different IVM protocols. No statistical difference of the indices used for fertilization, cleavage, and embryonic development in the present study was found between the in vitro-matured oocytes from MI stage oocytes with Standard IVM protocol and the corresponding in vitro-matured oocytes with autologous mature FF IVM protocol. A similar situation was found between the in vitro-matured oocytes from the GV stage oocytes with the Standard IVM protocol and the corresponding in vitro-matured oocytes with autologous mature FF & cumulus cell coIVM.

FIGURE 1

Cumulative time course of in vitro maturation (IVM) for cumulus-removed human oocytes at metaphase I stage of meiosis under two different IVM protocols. a = compared with the corresponding data of autologous mature follicular fluid (MFF) IVM protocol, Fisher's exact test, $P=.0375$.

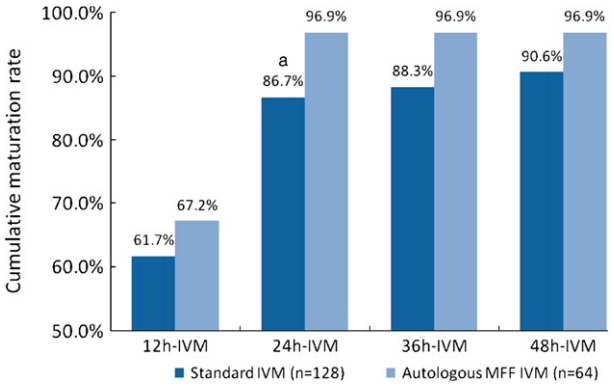

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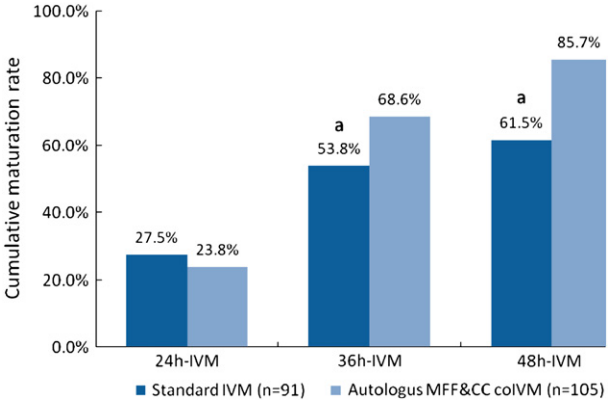
FIGURE 2

Cumulative time course of in vitro maturation (IVM) for cumulus-removed oocytes at germinal vesicle stage of meiosis under two different IVM protocols. a = compared with the corresponding data of autologous mature follicular fluid and autologous cumulus cell-assisted IVM co-culture; $\chi^2 > 3.381$, $P<.05$. Autologous MFF&CC coIVM = both autologous mature follicular fluid and autologous cumulus cell-assisted IVM co-culture protocols.



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Fertilization, Cleavage, and Preimplantation Development of In Vitro-Matured Oocytes at Different IVM Durations

In the present study, most (122/192, 63.5%) of MI stage oocytes matured in 12 hours in IVM and most (121/196, 61.7%) of GV stage oocytes matured in 36 hours in IVM. To exclude the possibility that developmental potential of immature oocytes subjected to ICSI at a certain time point after IVM culture is being masked by lack of a time-effect at other time points, the relevant developmental potential (at 12 hours after IVM culture for MI stage oocytes and at 36 hours after IVM culture for GV stage oocytes) were analyzed. Table 2 shows the fertilization, cleavage, and embryonic development of in vitro-matured oocytes at different IVM durations. No statistical difference was found in all the indices for fertilization, cleavage, eight-cell embryo development at day 3, good quality embryo development at day 3, or blastocyst development at day 5 between in vitro-matured oocytes from MI stage oocytes undergoing ≤ 12 hours of IVM culture and the in vitro-matured oocytes from MI stage oocytes undergoing >12 hours of IVM culture ($P>.05$). A similar situation was found between the in vitro-matured oocytes from GV stage oocytes undergoing ≤ 36 hours of IVM culture

TABLE 1

Comparison of fertilization, cleavage, and embryonic development of oocytes matured through different IVM protocols.

Oocyte classification	Fertilization		Cleavage		8-cell at D3		Good quality embryos at D3[a]		Blastocyst at D5	
	No.	%	No.	%	No.	%	No.	%	No.	%
In-vitroMOs from MI stage oocytes										
Matured through Standard IVM	53/106	50.0	41/53	77.4	13/41	31.7	7/41	17.1	3/41	7.3
Matured through Autologous MFF IVM	28/55	50.9	23/28	82.1	7/23	30.4	5/23	21.7	2/23	8.7
χ^2 test or Fisher's exact test	$P>.05$		$P>.05$		$P>.05$		$P>.05$		$P>.05$	
In-vitroMOs from GV stage oocytes										
Matured through Standard IVM	18/49	36.7	8/18	44.4	1/8	12.5	0/8	0.0	0/8	0.0
Matured through Autologous MFF& CC coIVM	37/79	46.8	23/37	62.2	4/23	17.4	0/23	0.0	0/23	0.0
χ^2 test or Fisher's exact test	$P>.05$		$P>.05$		$P>.05$		$P>.05$		$P>.05$	

[a] The embryo that shows ≥8 blastomeres at day 3 after intracytoplasmic sperm injection (ICSI) with uniformity or slight irregularity in blastomere morphology, less than 10% of anucleated fragmentation occupying the embryo volume and nongranular cytoplasm; IVM = in vitro maturation; In-vitroMOs = in vitro-matured oocytes; MI stage = metaphase of meiosis I; GV stage = germinal vesicle stage of meiosis; Autologous MFF IVM = autologous mature follicular fluid-assisted IVM protocol; Autologous MFF&CC coIVM = the IVM co-culture protocol assisted by both autologous mature follicular fluid and autologous cumulus cells.

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and the in vitro-matured oocytes from GV stage oocytes undergoing >36 hours of IVM culture.

Fertilization, Cleavage, and Embryonic Development of In Vivo- and In Vitro-Matured Oocytes

Figure 3 shows the fertilization, cleavage, and embryonic development of in vivo- and in vitro-matured oocytes. All of the indices used for fertilization, cleavage, and embryo development of autologous in vivo-matured oocytes were significantly higher than those of autologous in vitro-matured oocytes from either GV or MI stage oocytes. The rate of cleavage and the rate of good quality embryo development at day 3 of in vitro-matured oocytes from MI stage oocytes were also significantly higher than those of in vitro-matured oocytes from GV stage oocytes.

DISCUSSION

In the present study, two IVM culture systems with the assistance of either autologous mature FF alone (autologous mature FF IVM) or both autologous mature FF and autologous cumulus cells (autologous mature FF and cumulus cell coIVM) were developed to assess the effects of autologous mature FF on the IVM of human cumulus-removed oocytes, particularly on the IVM co-culture with autologous cumulus cells. These two IVM culture systems were specifically for the IVM of human MI and GV stage cumulus-removed oocytes. To our knowledge, this is the first research report of this aspect in humans.

In the present study, autologous mature FF was supplemented into the two IVM culture systems. Compared with the Standard IVM protocol, the promoting effect of the two IVM culture systems on the IVM of corresponding oocytes was evident. The maturation of oocytes proceeded more rapidly and resulted in a higher cumulative maturation rate in either the autologous mature FF IVM or the autologous mature FF & cumulus cell coIVM. The difference in the present study in the IVM outcome is in the participation of autologous mature FF and autologous cumulus cells into the IVM culture system, especially the participation of autologous mature FF.

It is generally accepted that the presence of granulosa cells (GCs), either mural GC or cumulus cells, is beneficial for human oocyte maturation (1, 2, 6, 8, 13). The mechanism by which co-culture of immature oocytes with GCs results in an improved IVM outcome has not been clearly identified. Nevertheless, two major common hypotheses have been proposed (35). First, co-culture of immature oocytes with GCs may alter the chemical composition of IVM medium by

TABLE 2

Comparison of fertilization, cleavage, and embryonic development of in vitro-matured oocytes in different IVM durations.

Oocyte classification	Fertilization		Cleavage		8-cell at D3		Good quality embryos at D3[a]		Blastocyst at D5	
	No.	%	No.	%	No.	%	No.	%	No.	%
In-vitroMOs from MI stage oocytes										
≤12 h in IVM	59/111	53.2	48/59	81.4	16/48	33.3	10/48	20.8	5/48	10.4
>12 h in IVM	22/50	44.0	16/22	72.7	4/16	25.0	2/16	12.5	0/16	0.0
χ^2 test or Fisher's exact test	$P>.05$		$P>.05$		$P>.05$		$P>.05$		$P>.05$	
In-vitroMOs from GV stage oocytes										
≤36 h in IVM	49/106	46.2	28/49	57.1	5/28	17.9	0/28	0.0	0/28	0.0
>36 h in IVM	6/22	27.3	3/6	50.0	0/3	0.0	0/3	0.0	0/3	0.0
χ^2 test or Fisher's exact test	$P>.05$		$P>.05$		$P>.05$		$P>.05$		$P>.05$	

[a] The embryo that shows ≥8 blastomeres at day 3 after ICSI with uniformity or slight irregularity in blastomere morphology, less than 10% of anucleated fragmentation occupying the embryo volume and nongranular cytoplasm; IVM = in vitro maturation; in-vitroMOs = in vitro-matured oocytes; MI stage = metaphase of meiosis I; GV stage = germinal vesicle stage of meiosis.

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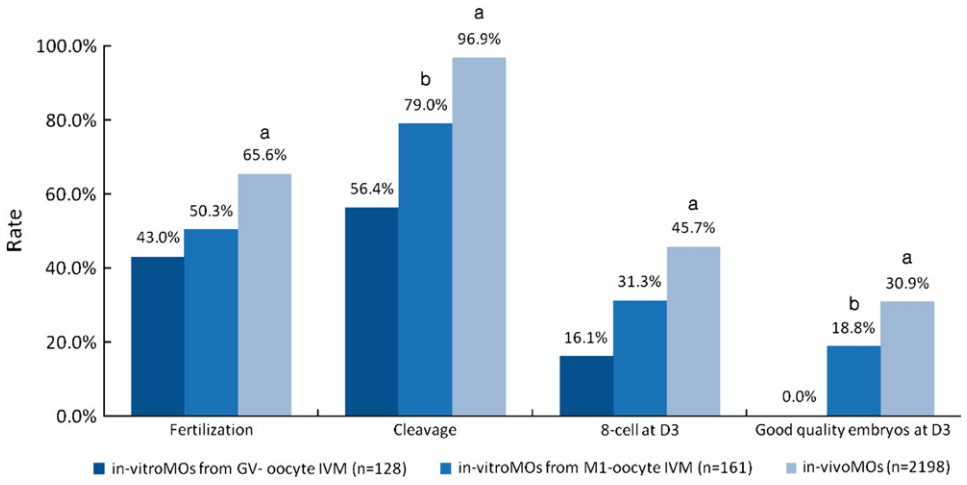
altering the concentration of metabolites, removing undesirable factors or detoxifying the IVM medium (12, 36). Second, the added GCs may contribute maturation-related factors or signals that directly affect the IVM of oocytes (9, 11–13, 21). Thus, IVM co-culture with GCs, especially IVM co-culture with autologous ones, was raised specifically to assist the IVM of human oocytes. Although some fairly encouraging results of IVM co-culture with heterologous monkey kidney cells (7) or homologous cumulus cells (15) have been obtained in humans, the available data on the IVM co-culture with autologous GCs did not demonstrate significant improvement in the IVM of human oocytes (5, 16).

In the present study, adding 10% (v/v) autologous mature FF into the IVM medium was the main modification compared with the previously developed co-culture system in the oocyte IVM (5, 16). This modification was mainly based on the accumulating evidence that human mature FF contains high levels of various components that are helpful for oocyte maturation and embryo development (18–21, 37). This modification was also based on the previous research results demonstrating that the promotion effects of human mature FF, as a heterologous or homologous supplement in the culture medium, on the meiotic resumption of mouse oocytes (22) and the IVM of human oocytes in the form of the cumulus–oocyte complex (24). Consistent results with these previous research results were obtained in the present study, in which the promoting effects of both IVM culture systems on the IVM of human oocytes were demonstrated. This means that the supplement of a certain amount of autologous mature FF to the IVM medium does favor the IVM of human cumulus-removed oocytes, particularly the IVM co-culture with autologous cumulus cells. The favorable effects of supplemented autologous MFF are not only from the immediate and direct support of autologous mature FF to IVM, but also from the possible stimulating effects of autologous mature FF on the growth of concurrently cultured autologous cumulus cells, the indirect support to IVM.

In the present study the importance of stimulating effects of autologous mature FF on the growth of concurrently cultured autologous cumulus cells in the IVM co-culture system for the IVM of GV stage human oocytes should be emphasized. The stimulating effect of autologous mature FF was presumed to be similar to the results reported by Somigliana and colleagues (30). In their study, a significant enhanced ability to stimulate proliferation of human endometrial cell growth in vitro was observed by using human mature FF similar to the one used in the present study. In the present study, we have hypothesized that the possible reasons for the insignificant improvement in the IVM co-culture of oocytes with autologous cumulus cells alone (5, 16) was the relatively delayed growth (i.e., the delayed feeder layer cell state) of autologous cumulus cells at the initial period of IVM. The presumed stimulating effects of autologous mature FF on the growth of concurrently cultured autologous cumulus cells may correct, at least partially, the relatively delayed growth of autologous cumulus cells and promote the concurrently cultured autologous cumulus cells to enter the state of feeder layer cells more quickly and to exert their positive impact in time on the IVM of human oocytes. The results obtained in the present study corroborate the hypothesis and, for the first time, provide evidence that the beneficial effects of autologous mature FF are contributing to the positive impact of

FIGURE 3

Fertilization, cleavage, and preimplantation embryonic development of sibling in vivo-matured oocytes (in-vivoMOs) and in vitro-matured oocytes (in-vitroMOs). a = compared with the corresponding data of sibling in vitro-matured oocytes matured from either germinal vesicle (GV) or metaphase I (M1) stage oocytes, χ^2 test or Fisher's exact test, $P<.05$. b = compared with the corresponding data of sibling in vitro-matured oocytes from germinal vesicle stage oocytes, χ^2 test or Fisher's exact test, $P<.05$. GV stage = germinal vesicle stage of meiosis. MI stage = metaphase of meiosis I. Good quality embryos at D3 = those embryos that showed ≥8 blastomeres at day 3 after intracytoplasmic sperm injection with uniformity or slightly irregularity in blastomere morphology, less than 10% of anucleate fragmentation occupying the embryo volume, and nongranular cytoplasm.



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two newly developed IVM culture systems on the IVM of human cumulus-removed oocytes.

Unfortunately, as shown in the present study, the obvious IVM promotion on the human oocytes with either autologous mature FF alone or both autologous mature FF and autologous cumulus cells was not accompanied with the corresponding improvement in the subsequent fertilization and embryonic development. This asynchronous result inferred that the improving effects of two newly developed IVM culture systems on human oocytes were limited and targeted mainly nucleus maturation, rather than cytoplasmic maturation.

Regarding the asynchrony of nucleus and cytoplasmic maturation, a common problem in IVM of human oocytes (1–3, 38), likely reasons for it might be the different maturation mechanisms, suboptimal IVM culture conditions for cytoplasmic maturation, or the comparatively serious intrinsic deficiencies of oocytes in cytoplasmic maturation compared with those of nuclear maturation (5, 38–40). It has been known that insufficient or improper cytoplasmic maturation of the oocyte will fail to complete the normal fertilization and development. Further studies are needed to optimize the related IVM.

In the present study, the comparatively systematic data on the IVM of human immature oocytes also showed that, although it is probable for most of human immature oocytes from routine stimulated ovaries to mature with IVM culture, the GV stage oocytes demonstrated a greatly lower IVM rate (Figs. 1 and 2) and an inferior fertilization and developmental potential (Fig. 3) compared with those of MI stage oocytes. Furthermore, our data showed that, in vitro-matured oocytes, from either GV or MI stage oocytes, did not exhibit similar fertilization and developmental potential compared with their in vivo-matured counterparts (Fig. 3). A lower frequency of fertilization and a higher frequency of cleavage arrest beyond the 4- to 8-cell stage of human embryos derived from in vitro-matured oocytes were observed. This situation was especially pronounced in the in vitro-matured oocytes derived from GV stage oocytes. No statistical difference in fertilization, cleavage, and preimplantation embryonic development of in vitro-matured oocytes was found at different IVM durations (Table 2). These results fit with the results shown in the previous relevant studies of this aspect (3, 4, 38, 41, 42), and, furthermore, corroborate the existing viewpoint that the immature state of oocytes did infer to some extent of inherent deficiencies of the corresponding oocytes in in vivo, and possibly further in vitro, development and maturation (5, 29, 39–44). It seems that the more delayed the in vivo development and maturation of an oocyte, the worse the IVM outcome and subsequent developmental potential of the oocyte.

In conclusion, adding a certain amount of autologous mature FF to the medium of the IVM system favors the IVM of human cumulus-removed oocytes, including the IVM co-

culture with autologous cumulus cells. Autologous mature FF makes the IVM culture system more physiological, synchronous, and effective. The two IVM culture systems, autologous mature FF IVM system and autologous mature FF & cumulus cell coIVM are both safe from the risk of pathogen transmission, superior for the rescue IVM of human oocytes, and suitable for the IVM of MI and GV stage cumulus-removed oocytes. They allow additional human oocytes to mature in vitro and to use in clinic or research work. The beneficial effects of the two IVM culture systems on human cumulus-removed oocytes are limited, mainly at the corresponding nuclear maturation. Further studies are needed to optimize each of the two IVM culture systems.

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REFERENCES

1. Mikkelsen AL. Strategies in human in-vitro maturation and their clinical outcome. Reprod Biomed Online 2005;10:593–9.
2. Jurema MW, Nogueira D. In vitro maturation of human oocytes for assisted reproduction. Fertil Steril 2006;86:1277–91.
3. Cekleniak NA, Combelles CMH, Ganz DA, Fung J, Albertini DF, Racowsky C. A novel system for in vitro maturation of human oocytes. Fertil Steril 2001;75:1185–93.
4. Chian RC, Tan SL. Maturational and developmental competence of cumulus-free immature human oocytes derived from stimulated and intracytoplasmic sperm injection cycles. Reprod Biomed Online 2002;5:125–32.
5. Combelles CMH, Fissore RA, Albertini DF, Racowsky C. In vitro maturation of human oocytes and cumulus cells using a co-culture three-dimensional collagen gel system. Hum Reprod 2005;20:1349–58.
6. Goud PT, Goud AP, Qian C, Laverge H, Van der Elst J, De Sutter P, et al. In-vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium. Hum Reprod 1998;13:1638–44.
7. Janssenswillen C, Nagy ZP, Van Steirteghem A. Maturation of human cumulus-free germinal vesicle-stage oocytes to metaphase II by coculture with monolayer Vero cells. Hum Reprod 1995;10:375–8.
8. Hwang JL, Lin YH, Tsai YL. In vitro maturation and fertilization of immature oocytes: a comparative study of fertilization techniques. J Assist Reprod Genet 2000;17:39–43.
9. Byskov AG, Yding Andersen C, Hossaini A, Guoliang X. Cumulus cells of oocyte-cumulus complexes secrete a meiosis-activating substance when stimulated with FSH. Mol Reprod Dev 1997;46:296–305.
10. Cao X, Pomerantz SH, Popliker MT, Safriri A. Meiosis-activating sterol synthesis in rat preovulatory follicle: is it involved in resumption of meiosis? Biol Reprod 2004;71:1807–12.
11. Atef A, Francois P, Christian V, Marc-Andre S. The potential role of gap junction communication between cumulus cells and bovine oocytes during in vitro maturation. Mol Reprod Dev 2005;71:358–67.
12. Downs SM, Gilles R, Vanderhoef C, Humpherson PG, Leese HJ. Differential response of cumulus cell-enclosed and denuded mouse oocytes in a meiotic induction model system. Mol Reprod Dev 2006;73:379–89.
13. Gilchrist RB, Ritter LJ, Armstrong DT. Oocyte–somatic cell interactions during follicle development in mammals. Anim Reprod Sci 2004;82–83:431–46.
14. Farhi J, Nahum H, Zakut H, Levran D. Incubation with sperm enhances in vitro maturation of the oocyte from the germinal vesicle to the M2 stage. Fertil Steril 1997;68:318–22.
15. Torre ML, Munari E, Albani E, Levi-Setti PE, Villani S, Faustini M, et al. In vitro maturation of human oocytes in a follicle-mimicking three-dimensional coculture. Fertil Steril 2006;86:572–6.
16. Haberle M, Scheurer P, Lauerer K, Fischer M, Hohl MK. Are cumulus cells necessary for the spontaneous maturation of germinal vesicle-stage oocytes to metaphase II. J Assist Reprod Genet 1999;16:329–31.
17. Fabbri R, Porcu E, Marsella T, Primavera MR, Cecconi S, Nottola SA, et al. Human embryo development and pregnancies in a homologous granulosa cell coculture system. J Assist Reprod Genet 2000;17:1–12.
18. Artini PG, Battaglia C, D'Ambrogio G, Barreca A, Droghini F, Volpe A, et al. Relationship between human oocyte maturity, fertilization and follicular fluid growth factors. Hum Reprod 1994;9:902–6.
19. Malamitsi-Puchner A, Sarandakou A, Baka S, Vrachnis N, Kouskouni E, Hassiakos D. Soluble Fas concentrations in the follicular fluid and oocyte-cumulus complex culture medium from women undergoing in vitro fertilization: association with oocyte maturity, fertilization, and embryo quality. J Soc Gynecol Invest 2004;11:566–9.
20. Westergaard L, Byskov AG, Van Look PF, Angell R, Aitken J, Swanston IA, et al. Meiosis-inducing substances in human preovulatory follicular fluid related to time of follicle aspiration and to the potential of the oocyte to fertilize and cleave in vitro. Fertil Steril 1985;44:663–7.
21. Sutton ML, Gilchrist RB, Thompson JG. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus–oocyte complex and its influence on oocyte developmental capacity. Hum Reprod Update 2003;9:35–48.
22. Chi HJ, Kim DH, Koo JJ, Chang SS. The suitability and efficiency of human follicular fluid as a protein supplement in human in vitro fertilization programs. Fertil Steril 1998;70:871–7.
23. Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. Fertil Steril 1991;55:109–13.
24. Liu JY, Qian Y, Mao YD, Ding W, Yang NM. In vitro maturation, fertilization and embryo transfer of human immature oocyte. Zhonghua Fu Chan Ke Za Zhi 2003;38:230–2.
25. Ali A, Coenen K, Bousquet D, Sirard M-A. Origin of bovine follicular fluid and its effect during in vitro maturation on the developmental competence of bovine oocytes. Theriogenology 2004;62:1596–606.
26. Avery B, Strobech L, Jacobsen T, Bogh IB, Greve T. In vitro maturation of bovine cumulus–oocyte complexes in undiluted follicular fluid: effect on nuclear maturation, pronucleus formation and embryo development. Theriogenology 2003;59:987–99.
27. Bogh IB, Bezard J, Duchamp G, Baltsen M, Gerard N, Daels P, et al. Pure preovulatory follicular fluid promotes in vitro maturation of in vivo aspirated equine oocytes. Theriogenology 2002;57:1765–79.
28. Conforti VA, Vanderwall DK, Woods GL. Effect of homologous follicular fluid from medium-sized and large follicles on in vitro maturation of equine cumulus-oocyte complexes. Reprod Fertil Dev 2005;17:651–8.
29. Ng EHY, Lau EYL, Yeung WSB, Ho PC. HMG is as good as recombinant human FSH in terms of oocyte and embryo quality: a prospective randomized trial. Hum Reprod 2001;16:319–25.
30. Somigliana E, Vigano P, La Sala GB, Balasini M, Candiani M, Incerti L, et al. Follicular fluid as a favourable environment for endometrial and endometriotic cell growth in vitro. Hum Reprod 2001;16:1076–80.
31. World Health Organization. WHO laboratory manual for the examination of human semen and sperm–cervical mucus interaction. 4th ed. Cambridge: Cambridge University Press, 1999.
32. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 1992;340:17–8.
33. Huang F-J, Lan K-C, Kung F-T, Tsai M-Y, Chang C-Y, Huang H-W, et al. Human cumulus-free oocyte maturational profile and in vitro developmental potential after stimulation with recombinant versus urinary FSH. Hum Reprod 2004;19:306–15.
34. Veeck L. An atlas of human gametes and conceptuses. New York: Parthenon, 1998.
35. Carrell DT, Peterson CM, Jones KP, Hatasaka HH, Udoff LC, Cornwell CE, et al. A simplified coculture system using homologous,

attached cumulus tissue results in improved human embryo morphology and pregnancy rates during in vitro fertilization. J Assist Reprod Genet 1999;16:344–9.
36. Joo BS, Kim MK, Na YJ, Moon HS, Lee KS, Kim HD. The mechanism of action of coculture on embryo development in the mouse model: direct embryo-to-cell contact and the removal of deleterious components. Fertil Steril 2001;75:193–9.
37. Grondahl C, Breinholt J, Wahl P, Murray A, Hansen TH, Faerge I, et al. Physiology of meiosis-activating sterol: endogenous formation and mode of action. Hum Reprod 2003;18:122–9.
38. Combelles CM, Cekleniak NA, Racowsky C, Albertini DF. Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes. Hum Reprod 2002;17:1006–16.
39. Combelles CM, Albertini DF, Racowsky C. Distinct microtubule and chromatin characteristics of human oocytes after failed in-vivo and in-vitro meiotic maturation. Hum Reprod 2003;18:2124–30.
40. Mrazek M, Fulka J Jr. Failure of oocyte maturation: possible mechanisms for oocyte maturation arrest. Hum Reprod 2003;18:2249–52.
41. Kim B-K, Lee S-C, Kim K-J, Han C-H, Kim J-H. In vitro maturation, fertilization, and development of human germinal vesicle oocytes collected from stimulated cycles. Fertil Steril 2000;74:1153–8.
42. Strassburger D, Friedler S, Raziel A, Kasterstein E, Schachter M, Ron-El R. The outcome of ICSI of immature MI oocytes and rescued in vitro matured MII oocytes. Hum Reprod 2004;19:1587–90.
43. Li Y, Feng HL, Cao YJ, Zheng GJ, Yang Y, Mullen S, et al. Confocal microscopic analysis of the spindle and chromosome configurations of human oocytes matured in vitro. Fertil Steril 2006;85:827–32.
44. Emery BR, Wilcox AL, Aoki VW, Peterson CM, Carrell DT. In vitro oocyte maturation and subsequent delayed fertilization is associated with increased embryo aneuploidy. Fertil Steril 2005;84:1027–9.