Human Reproduction vol.13 no.6 pp.1638–1644, 1998

In-vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium

P.T.Goud[1], A.P.Goud, C.Qian, H.Laverge, J.Van der Elst, P.De Sutter and M.Dhont

Infertility Centre, Department of Obstetrics and Gynaecology, University Hospital, Ghent, Belgium

[1]To whom correspondence should be addressed

In-vitro maturation (IVM) of oocytes is a promising technique to reduce the costs and avert the side-effects of gonadotrophin stimulation for in-vitro fertilization (IVF). The pregnancy rates from oocytes matured *in vitro* are much lower than those of in-vivo stimulation cycles indicating that optimization of IVM remains a challenge. Therefore, we investigated the effect of supplementation of the medium with gonadotrophins, oestradiol and epidermal growth factor (EGF) and the effect of retaining or removing the cumulus cells on nuclear and cytoplasmic maturation of immature oocytes. Human germinal vesicle (GV) oocytes obtained after gonadotrophin stimulation for intracytoplasmic sperm injection (ICSI) were cultured in a complex defined medium either supplemented with gonadotrophins, oestradiol and physiological concentrations of EGF (2 ng/ml) or gonadotrophins and oestradiol alone. The cumulus cells were either removed or kept intact. In GV stage oocytes cultured without cumulus (group I) significantly more oocytes reached the metaphase II (MII) stage at 30 h in media supplemented with EGF (64.3 versus 33.9%, $P < 0.003$). For oocytes cultured with intact cumulus (group II), more oocytes reached MII at 30 h than in group I, but there was no difference in medium with or without EGF supplementation (81.8 and 79.8% respectively). Cytoplasmic maturation of MII oocytes was judged from their capability to activate and fertilize after ICSI. In group I, the rates of activation and normal fertilization were similar. However, in group II, significantly more oocytes underwent normal fertilization in the EGF-supplemented than the unsupplemented group (71.7 versus 45.6%, $P < 0.05$). The cleavage rates of the fertilized oocytes were similar in the sibling oocyte subgroups cultured with or without EGF supplementation, but the overall cleavage rates were higher in cumulus-intact compared to cumulus-denuded oocytes (88.9 versus 47.8%, $P < 0.001$). Thus, supplementation of the maturation medium with EGF and maintenance of the cumulus during culture improve the nuclear and cytoplasmic maturation of human oocytes *in vitro*.

Key words: epidermal growth factor/maturation *in vitro*/ICSI/oocyte

Introduction

In-vitro maturation (IVM) of human oocytes would be an attractive alternative to gonadotrophin stimulation for in-vitro fertilization (IVF). However, the efficiency of current IVM techniques is suboptimal in terms of the number of mature oocytes available per cycle in comparison to gonadotrophin stimulated cycles (Barnes *et al*., 1995; Russell *et al*., 1997). Also, the quality of maturation appears to be suboptimal because embryos resulting from in-vitro matured oocytes show more frequent cleavage blocks and overall retarded cleavage rates as compared to their in-vivo matured counterparts (Barnes *et al*., 1996; Trounson *et al*., 1996). Therefore, in order to make the technique acceptable and clinically applicable, improvement is needed on both fronts. Therefore the strategy for increasing the efficiency of IVM needs to be focused primarily on optimizing the oocyte retrieval technique and developing an optimal culture system for IVM.

Fairly successful culture conditions have been devised for IVM in bovine and some other species (Fukuda *et al*., 1990; Bavister *et al*., 1992; Eppig *et al*., 1996). However, interspecies differences in the maturation physiology, especially in the final stages of maturation (Edwards, 1965a,b), do not allow their direct extrapolation to the human system. Therefore, appropriate culture conditions specifically for maturing human oocytes are essential. Although recently the technique of IVM has been applied clinically with some limited success (Cha *et al*., 1991; Trounson *et al*., 1994; Barnes *et al*., 1995, 1996), most of these culture systems employ complex media supplemented with serum. Defining the culture conditions is difficult since serum may contain many unknown substances in addition to albumin, amino acids and certain other factors including growth factors (Bavister, 1995). We chose therefore to mature human oocytes in medium without serum.

Epidermal growth factor (EGF) plays a positive role in various mammalian in-vitro systems of oocyte maturation (Downs, 1989; Harper and Bracket, 1993; Coskun and Lin, 1995; Lonergan *et al*., 1996; Lorenzo *et al*., 1996). Although a similar beneficial influence of EGF has also been suggested in the human (Das *et al*., 1991; Gomez *et al*., 1993), little is known of the role of EGF in human IVM in the presence or absence of the cumulus cells and in the presence of gonadotrophins. Therefore, we added EGF to our culture system to test its influence on human oocyte maturation in the presence or absence of the cumulus cells.

Obtaining germinal vesicle (GV) stage human oocytes from unstimulated ovaries for experimental use has ethical and legal limitations apart from the special expertise required at retrieval (Trounson *et al*., 1994). On the other hand, GV oocytes

obtained during an IVF/intracytoplasmic sperm injection (ICSI) cycle are not used clinically and are usually discarded. Although some of these oocytes may be atretic or may have been resistant to the in-vivo gonadotrophin stimulus, many are still capable of undergoing maturation and fertilization, if appropriate conditions are present *in vitro* (Janssenswillen *et al*., 1995). Hence, these oocytes could be used to study and optimize the culture conditions for the human IVM.

Oocytes from different patients may be diverse in characteristics and quality. Therefore, they may not be suitable for randomized comparisons. Sibling immature oocytes may be more similar in their physiological characteristics and quality. Therefore, we randomized sibling oocytes with similar morphological characteristics and cumulus cell status to compare their nuclear and cytoplasmic maturation after in-vitro culture.

The oocytes were randomized between two maturation media, one of which was supplemented with human recombinant EGF at a physiological concentration (2 ng/ml), close to that in the follicular fluid (Westergaard and Andersen, 1989; Hoffmann *et al*., 1990). The nuclear and cytoplasmic maturation of the cultured oocytes was judged from their capability to undergo germinal vesicle breakdown (GVBD), polar body extrusion, fertilization and early cleavage after ICSI.

Materials and methods

Study design

Only oocytes from patients having two or more spare GV oocytes with similar morphological features and intact cumulus cells were selected for the study. After confirming the presence of GV, the cumulus cells were either totally removed or kept intact according to the experimental set. Sibling oocytes of similar size, cumulus cell status and appearance were randomized between two maturation media supplemented with gonadotrophins oestradiol and EGF or gonadotrophins and oestradiol alone. Nuclear maturation was determined by examining the oocytes at 12, 24 and 30 h for GVBD and polar body extrusion. Oocytes reaching metaphase II (MII) were injected with donor spermatozoa and the culture of the injected oocytes was continued further to investigate the activation, fertilization and first cleavage competence of the oocytes.

Chemicals, reagents and culture media

Medium 199 (M-199) was obtained from Gibco BRL (N.V. Life Technologies, Brussels, Belgium). Human serum albumin (HSA, Albumine 20%) was obtained from the Belgian Red Cross (Brussels, Belgium). All other chemicals and reagents were obtained from Sigma (St Louis, MO, USA), unless specified otherwise.

The maturation medium used for the culture of immature oocytes (medium I) was M-199 supplemented with 0.4% HSA, 0.29 mM pyruvate, 0.05 mg/ml penicillin, 0.075 mg/ml streptomycin, 1 μg/ml 17β-oestradiol, 0.075 IU/ml follicle stimulating hormone (FSH, Metrodin HP; Serono Benelux, Brussels, Belgium) and 0.5 IU/ml human chorionic gonadotrophin (HCG, Profasi; Serono Benelux, Brussels, Belgium) as described before (Barnes *et al*., 1995; Russell *et al*., 1997). EGF was reconstituted to 1 mg/ml in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (w/v) and stored in 10 μl aliquots at –20°C. One of the two media in each group (medium II) was supplemented with 2 ng/ml of recombinant EGF in addition to all the other components mentioned above. The final concentration of BSA in the EGF supplemented culture medium was 0.0001%.

Source of gametes

The project involving IVM and fertilization of spare GV oocytes with donor spermatozoa for research was sanctioned by the institutional ethical review committee. The oocytes were obtained from 92 patients undergoing ICSI cycles at our centre. Prior informed consent was obtained from the patients to mature and inject their spare GV oocytes with donor spermatozoa, having explained to them the experimental nature of the work. Ovarian stimulation was performed using a gonadotrophin-releasing hormone agonist (GnRHa, Decapeptyl; Ipsen Biotech, Paris, France) in a long protocol and human menopausal gonadotrophin (HMG, Humegon; Organon Belgium S.A., Brussels, Belgium; Metrodin HP; Serono Benelux; Menogon, Ferring arzneimittel GmbH, Kiel, Germany) given daily, starting from day 2 or 3. Alternatively, GnRHa was used in a short protocol in which HMG was started on day 2 or 3 of the cycle. In both cases, stimulation was continued until the leading follicles achieved an optimum size of 20 mm as confirmed by transvaginal ultrasound, after which 10 000 IU HCG (Pregnyl; Organon) was administered. The oocyte retrieval was performed 34–36 h post HCG, with ultrasound-guided needle puncture. Spermatozoa donated for research were used for ICSI of the in-vitro matured oocytes.

Randomization, culture and examination of the oocytes

All the oocytes obtained from the follicular aspirates were treated with 0.1% hyaluronidase for 1–2 min as per our routine protocol for ICSI. Following this, the presence of the GV could be easily judged in most of the immature oocytes, using Hoffman contrast optics and a magnification of ×200–400 even in the presence of one or more layers of the cumulus corona cells (Figure 1A). The findings were reconfirmed by a second observer independently before assigning the oocyte as 'GV stage'. Therefore, further removal of cumulus cells was rarely necessary just for visualization of the GV. However, in case of the latter, inclusion of such oocytes in the study was avoided except when the cumulus cell status prior to removal was similar in the oocytes assigned to both groups and the same procedure of cumulus cell removal had been performed on sibling oocytes in both groups.

In group I, 121 oocytes from 38 patients were completely denuded of their cumulus cells and then the sibling oocytes were randomized for culture at 37°C under 5% CO_2 in air in pre-equilibrated 25 μl drops of either maturation medium I (without EGF) or II (with EGF). In group II, the cumulus cells were retained on 191 GV oocytes from 54 patients which were then randomized for culture in medium I or II in a similar way. The oocytes were observed under an inverted microscope equipped with Hoffman contrast optics at 12, 24 and 30 h post oocyte retrieval. Special attention was given to GVBD and polar body extrusion. In group II, the cumulus cells were denuded at 24 h to determine the polar body status.

ICSI, embryo culture and cleavage assessment

ICSI was performed on 35 oocytes matured in group I and 92 oocytes in group II. In the latter, an equal number of sibling oocytes was injected in each subgroup. The technique of ICSI was similar to that described before (Dozortsev *et al*., 1996). After ICSI, the injected oocytes were rinsed and transferred to 25 μl drops of pre-equilibrated embryo culture media (Earle's balanced salt solution supplemented with 0.4% HSA, 0.3 mM pyruvate, 0.06 mg/ml penicillin G and 0.05 mg/ml streptomycin) under paraffin oil. Pronuclear status was checked 14–18 h post ICSI and the 2-pronuclear zygotes were cultured for another 24 h to investigate cleavage.

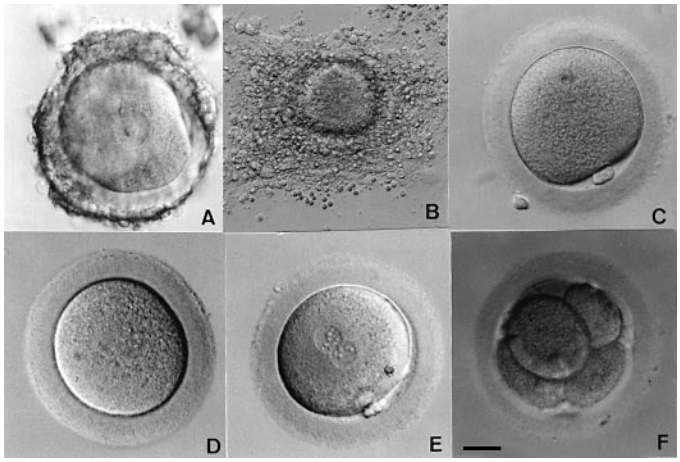

Figure 1. In-vitro maturation, fertilization, and cleavage of human germinal vesicle (GV) oocytes. (**A**) Human GV oocyte. The attached cumulus–corona cells can be seen. (**B**) Cumulus cell expansion and (**C**) completion of the second meiotic division after in-vitro culture. (**D**) An oocyte arrested in metaphase I. (**E**) A fertilized 2-pronuclear zygote (**F**). A cleaved embryo at 42 h post intracytoplasmic sperm injection. All pictures taken under Hoffman contrast optics. Original magnification of **A**, **C**, **D**, **E**, **F**: ×320; **B**: ×200. Bar = 32 μm.

Immunoblot analysis

The human serum albumin preparation was tested for contaminating EGF with immunoblot analysis. Immunoblots were performed using the enhanced chemiluminescence (ECL) system. The antibody concentrations were determined with the help of dot blots. Quantities of EGF up to 1 ng could be detected at the primary antibody (monoclonal anti-human EGF purified mouse immunoglobulin) concentration of 1:100, secondary antibody (mouse Ig horseradish peroxidase-linked whole antibody; Amersham, Ghent, Belgium) concentration of 1:2000 and an exposure time of 5 min. A sample corresponding to 2 mg HSA was run against a positive control of EGF (1 ng) on a one-dimensional, non-denaturing 15% sodium dodecyl sulphate–polyacrylamide gel. The proteins on the gel were subsequently transferred onto Hybond-ECL nitrocellulose membrane (Amersham) using a Western blotting apparatus. The blot was treated with a 5% blocking solution (non-fat dried milk powder) for 1 h followed by incubation with primary antibody for 90 min and secondary antibody for 1 h. The nitrocellulose membrane was then treated for 1 min with the reagent mixture (ECL system; Amersham) and exposed for 1 s–15 min to an X-ray film (Hyperfilm ECL; Amersham).

Statistical methods

Statistical analysis was performed by the paired Student's t test for sibling oocyte subgroups within groups I and II (Prism graphpad software®, San Diego, CA, USA). Comparison of the non-sibling oocyte subgroups was performed using the Fisher's exact test for 2×2 contingency table.

Results

The mean age of the patients was 31.9 years in the cumulus-denuded group (group I) and 31.8 years in the cumulus-intact group (group II). Other patient characteristics, including duration of infertility, cause of infertility (in addition to the male factor), stimulation protocol and number of ampoules of HMG/FSH were similar in both groups. Ninety-two patients had two or more GV oocytes at retrieval. The total number of oocytes obtained from these patients was 1505, of which 326 oocytes (21.7%) were found to be at GV stage. Of these, 121 GV oocytes were available for randomization in group I and 194 were available in group II. Eleven oocytes were discarded due to their grossly abnormal appearance.

Finally, in group I, 112 cumulus-denuded oocytes from 38 patients were actually suitable for a sibling oocyte comparison between medium I (no EGF, $n = 56$) and medium II (with EGF, $n = 56$) and in group II, 177 oocytes from 54 patients were suitable for a randomized sibling oocyte comparison between medium I (no EGF, $n = 89$) and medium II (EGF, $n = 88$). The other nine oocytes in group I and 17 oocytes in group II were judged to be unsuitable for comparison either due to their abnormal characteristics (e.g. abnormal cytoplasm appearance, vacuoles etc.) or due to their grossly dissimilar appearance in comparison to their sibling GV oocyte (e.g. grossly different size, different status of cumulus cells etc.). The maturational status of the oocytes at each time point is given in Tables I and II.

Germinal vesicle breakdown and polar body extrusion

In group I, the rate of GVBD at all the intervals was similar in both media (Table I). At 30 h, the rate of GVBD was 75% in medium I and in 89.3% in medium II. The rates of polar body extrusion were similar at 24 h, but at 30 h they were significantly higher in medium II, which was supplemented with EGF (Table I). In group II the rates of GVBD and polar body extrusion were similar in medium I and medium II; however, the number of oocytes remaining at MI at 30 h (Figure 1D) was higher in medium I (without EGF) (Table II). The proportion of MII oocytes at 30 h (Figure 1B,C) in group II was higher than for group I (Figure 2).

Oocyte activation and fertilization following ICSI

In both groups together, 127 oocytes were injected with spermatozoa. The number of oocytes undergoing activation, normal or abnormal fertilization and cleavage are presented (Table III). In group I, the numbers of oocytes undergoing activation and normal fertilization were similar. But in group II, significantly more oocytes underwent normal fertilization in the EGF-supplemented group (Table III, Figure 1E). Overall,

Table I. In-vitro maturation of cumulus-denuded oocytes cultured in maturation medium with or without epidermal growth factor (EGF)

Time	Maturation medium without EGF (n = 56)				Maturation medium with EGF (n = 56)			
	GV (%)	MI (%)	MII (%)	GVBD (%)	GV (%)	MI (%)	MII (%)	GVBD (%)
12 h	18 (32.1)	38 (67.8)	0	38 (67.8)	14 (25)	42 (75)	0	42 (75)
24 h	14 (25)	34 (60.7)	8 (14.3)	42 (75)	8 (14.3)	34 (60.7)	14 (25)	48 (85.7)
30 h	14 (25)	23 (41.1)	19 (33.9)[a]	42 (75)	6 (10.7)	14 (25)	36 (64.3)[a]	50 (89.3)

[a]$P < 0.003$.
GV = germinal vesicle; MI = metaphase I; MII = metaphase II; GVBD = germinal vesicle breakdown.

Table II. In-vitro maturation of cumulus-intact germinal vesicle human oocytes in maturation medium with or without epidermal growth factor (EGF) supplementation

Time	Maturation medium without EGF (n = 89)				Maturation medium with EGF (n = 88)			
	GV (%)	MI (%)	MII (%)	GVBD (%)	GV(%)	MI (%)	MII (%)	GVBD (%)
12 h	13 (14.6)	76 (85.4)	0	76 (85.4)	13 (14.8)	75 (85.2)	0	75 (85.2)
24 h	9 (10.1)	28 (31.5)	52 (58.4)	80 (89.9)	13 (14.8)	45 (51.1)	30 (34.1)	75 (85.2)
30 h	9 (10.1)	9 (10.1)[a]	71 (79.8)	80 (89.9)	13 (14.8)	3 (3.4)[a]	72 (81.8)	75 (85.2)

[a]$P < 0.04$.
For abbreviations see Table I.

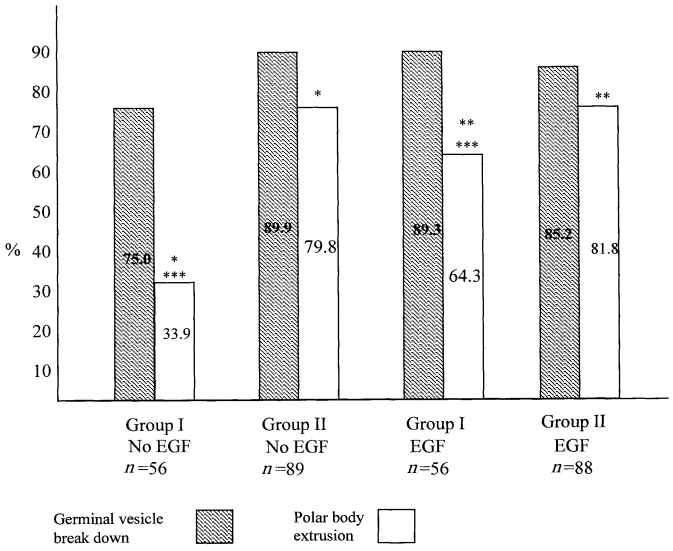

Figure 2. A bar chart to show the rates of germinal vesicle breakdown and polar body extrusion after 30 h culture in the different subgroups of oocytes (see Table III for definition of groups). *$P < 0.001$, **$P < 0.03$, ***$P < 0.003$.

a higher rate of cleavage (Figure 1F) was noted in group II as compared to group I (Table III); however, within group II, no difference was noted between the rates of cleavage of zygotes derived from subgroups of oocytes matured in either medium.

Immunoblot analysis

Results of the immunoblot analysis indicated that the albumin preparation used in our culture medium was free of contaminating EGF (Figure 3).

Discussion

Retrieval of immature human oocytes followed by IVM and fertilization has been recently performed with some limited success in clinical IVF/ICSI programmes (Cha *et al*., 1991, Trounson *et al*., 1994, 1996; Barnes *et al*., 1995, 1996; Russell *et al*., 1997). However, the pregnancy rates with IVM are very low in comparison to in-vivo matured oocytes obtained during gonadotrophin-stimulated cycles (Trounson *et al*., 1996). This can mainly be attributed to the relatively lower number of oocytes that mature, fertilize and finally develop successfully into embryos that implant. Nevertheless, it is worthwhile to carry on further investigations since IVM offers a number of advantages, such as prevention of hyperstimulation syndrome, reduced costs and relief from anxiety over the potential long-term side-effects of ovarian stimulation drugs. It also provides the opportunity to transfer the embryos during a natural cycle or during cycles that have minimal drug supplementation.

One of the most important factors regulating the number and quality of oocytes maturing *in vitro* is the culture system used for IVM. Culture media components and culture conditions can affect and even modulate the meiotic regulation of mammalian oocytes (Downs and Mastropolo, 1997; Kito and Bavister, 1997). It is therefore necessary to devise and optimize culture systems that take into account all the factors essential for the completion of oocyte maturation *in vitro*. The optimum design of most media used for human oocyte maturation is based mainly upon experience with other mammalian species. The most commonly used design has a culture medium supplemented with hormones and serum (Younis *et al*., 1989; Trounson *et al*., 1994). Serum, when added to the culture medium, acts as a source of albumin which balances the osmolality and scavenges potentially harmful molecules and metal ions that can act as a source of free oxygen radicals. Serum may also act as a source of growth factors and other beneficial substances that prevent premature loss of cortical granules and in-vitro zona hardening (Downs *et al*., 1986). Sometimes, serum may also have an inhibitory influence on the cytoplasmic maturation as seen in pig oocytes (Funahashi

Table III. Normal and abnormal fertilization and cleavage of in-vitro matured oocytes after intracytoplasmic sperm injection

Maturation medium and cumulus status	Cumulus-denuded oocytes (group I)			Cumulus-intact oocytes (group II)		
	No EGF	EGF	Total	No EGF	EGF	Total
Oocytes injected	13	22	35	46	46	92
2PN (%)	7 (53.8)	16 (72.7)	23 (65.7)	21 (45.6)[a]	33 (71.7)[a]	54 (58.7)
1PN (%)	0	1 (4.5)	1 (2.9)	4 (10.3)	2 (5.4)	6 (6.5)
3PN (%)	0	0	0	3 (6.5)	0	3 (3.3)
Total activation (%)	7 (53.8)	17 (77.3)	24 (68.6)	28 (60.9)	35 (76.1)	63 (68.5)
No activation (%)	6 (46.1)	5 (22.7)	11(31.4)	18 (39.1)	11 (23.9)	29 (31.5)
Cleavage (%)[c]	3 (42.8)	8 (50)	11(47.8)[b]	20 (95.2)	28 (84.8)	48 (88.9)[b]

PN = pronuclear; EGF = epidermal growth factor.
[a]$P < 0.05$; [b]$P < 0.001$; [c]values in parentheses are percentages among fertilized 2PN zygotes.

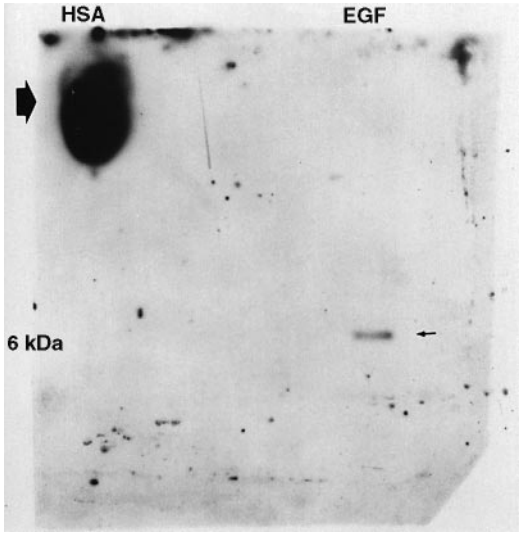

Figure 3. Western blot with human serum albumin (HSA) loaded in lane 1 against 1 ng epidermal growth factor (EGF) in lane 2. Note the absence in lane 1 of a signal for EGF corresponding to the positive control EGF band (thin arrow, ~6 kDa). Non-specific staining due to protein overload (thick arrow).

and Day, 1993). Finally, a number of unknown factors and contaminants may also be added to the medium along with serum. Thus, addition of serum greatly hampers the process of defining the culture in a research setting (Bavister, 1995). We preferred to supplement our culture medium with albumin rather than serum while studying the influence of EGF on the maturation of GV oocytes in the presence of hormones. The albumin preparation was pretested for contaminants and certified for clinical therapeutic use. We tested the albumin preparation further and found it to be free of EGF.

In group I, a direct positive effect of EGF was shown by a higher number of oocytes completing nuclear maturation in the medium supplemented with EGF in comparison with their sibling oocytes cultured without EGF. These oocytes were cultured in the absence of cumulus cells. The action of EGF, therefore, is likely to be on the oocyte itself, as was suggested by Lonergan *et al*. (1996) in bovine oocytes. The zona pellucida allows the passage of molecules as large as 150 kDa in the mouse (Legge, 1995). The human zona may be somewhat similar in this regard. Hence, the relatively small EGF molecule (~6 kDa) is likely to traverse easily through the zona of human oocytes to exert its effect directly on the oocyte. The presence of EGF receptors on the human oocyte and their up-regulation during the mid- follicular and the pre-ovulatory period further strengthen this possibility (Maruo *et al*., 1993). Thus, EGF may have exerted its positive effect directly on the oocyte after binding to its receptors and activating the tyrosine kinases (Gill, 1990).

A similar positive effect of EGF on the number of oocytes reaching the MII stage in culture was not demonstrated in the cumulus-intact oocytes. This may be due to the positive action of gonadotrophins through the cumulus cells, even in the absence of supplementary EGF (Moor and Trounson, 1977; Prins *et al*., 1987). However, the positive effect of EGF on cumulus-intact oocytes was evident at fertilization after ICSI. A relatively higher number of oocytes underwent normal fertilization in the EGF-supplemented group as compared to unsupplemented oocytes. Therefore, EGF is also likely to have a direct action on cytoplasmic maturation. No further beneficial effect was observed once cleavage had occurred. However, a positive effect of EGF supplementation on the later stages of embryo development (Harper and Bracket, 1993) was not studied and therefore cannot be ruled out. The effect of EGF thus appears to be synergistic with gonadotrophins in the cumulus-intact oocytes. Our findings are similar to those of Gomez *et al*. (1993) in terms of the positive effect of EGF on human oocyte maturation. However, we also found a positive effect of EGF on the rates of normal fertilization which was not studied by Gomez *et al*. (1993). Also, the positive effect of EGF supplementation on the rates of MII oocytes in their study was borderline significant at 2 ng/ml and became clearly evident only at 10 ng/ml.

In the cumulus-intact oocytes matured in the presence or absence of EGF, the rates of nuclear maturation were higher than the cumulus-denuded oocytes, irrespective of EGF supplementation. Even the overall cleavage rates in the cumulus-intact oocytes were higher, compared to cumulus-denuded oocytes cultured with or without EGF. The overall higher rates of maturation and cleavage indicate that the quality of cytoplasmic maturation was superior in the presence of the attached cumulus cells. The beneficial effects of cumulus cells on oocyte maturation and early development are well known in different species (mouse: Cross and Brinster, 1970; rat: Vanderhyden and Armstrong; 1989; human: Kennedy and

Donahue, 1969; ovine: Staigmiller and Moor, 1984; and bovine: Sirard *et al*., 1988; Fukui, 1990). The cumulus cells increase the surface area:volume ratio of the oocyte which increases the rate of entry of small molecules into the oocytes. The cumulus cells are also intimately connected with the oocyte through long microvilli that traverse through the zona to contact the oolemma to form gap junctions and desmosomes (Motta *et al*., 1994). The gap junctions help to mediate the transport of certain molecules that are necessary for oocyte metabolism (Brower and Schultz, 1982; Haghinat and Van Winkle, 1990). In addition to these effects, the granulosa cells also appear to exert some of their effects on the oocyte via paracrine signals which may explain the beneficial effect of co-culture of granulosa cells, especially when the latter were primed with FSH (Dandekar *et al*., 1991; Schramm and Bavister, 1996). A similar effect was also seen when cumulus-denuded oocytes were co-cultured with Vero cells (Janssenswillen *et al*., 1995). Recently, a novel 'apocrine' mechanism for the storage and release of growth factors and certain proteins was demonstrated in human follicular and cumulus cells (Antczak *et al*., 1997). Some of these proteins are expressed and selectively distributed in mature oocytes and developing embryos, which is suggestive of their role during embryogenesis (Antczak and Van Blerkom, 1997). These mechanisms may at least partly explain some of the beneficial effects of cumulus cells seen in our study.

Thus, our data indicate that EGF has a direct action on both cumulus-denuded and cumulus-intact oocytes during IVM in the human. Retention of the attached cumulus cells helps to achieve higher rates of cytoplasmic maturation as seen by the higher cleavage rates after fertilization. This information could guide us towards improvements in the existing IVM systems for human oocytes. However, further research needs to be directed to investigate factors influencing the quality of cytoplasmic maturation, which may be the reason for the high frequency of cleavage arrest beyond the 4–8-cell stage of human embryos derived from in-vitro matured oocytes.

Acknowledgements

The authors wish to thank Mr Georges Van Maele from the Department of Medical Informatics for his advice regarding the statistics.

References

Antczak, M. and Van Blerkom, J. (1997) Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation embryo. *Mol. Hum. Reprod.*, **3**, 1067–1086.

Antczak, M., Van Blerkom, J. and Clark, A. (1997) A novel mechanism of vascular endothelial growth factor, leptin and transforming growth factor-β2 sequestration in a subpopulation of human ovarian follicle cells. *Hum. Reprod.*, **12**, 2226–2234.

Barnes, F.L., Crombie, A., Gardner, D.K. *et al.* (1995) Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Hum. Reprod.*, **10**, 3243–3247.

Barnes, F.L., Kausche, A., Tiglias, J. *et al.* (1996) Production of embryos from *in vitro*-matured primary human oocytes. *Fertil. Steril.*, **65**, 1151–1156.

Bavister, B.D. (1995) Culture of preimplantation embryos: facts and artifacts. *Hum. Reprod. Update*, **1**, 91–148.

Bavister, B.D., Rose-Hellekant, T.A. and Pimyopummintr, T. (1992) Development of *in vitro* matured/*in vitro* fertilized bovine embryos into morulae and blastocysts in defined culture media. *Theriogenology*, **37**, 127–146.

Brower, P.T. and Schultz, R.M. (1982) Intercellular communication between granulosa cells and mouse oocytes: Existence and possible nutritional role during oocyte growth. *Dev. Biol.*, **90**, 144–153.

Cha, K.Y., Koo, J.J., Ko, J.J. *et al*. (1991). 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.*, **55**, 109–113

Coskun, S. and Lin, Y.C. (1995). Mechanism of action of epidermal growth factor-induced porcine oocyte maturation. *Mol. Reprod. Dev.*, **42**, 311–317.

Cross, P.C. and Brinster, R.L. (1970) In vitro development of mouse oocytes. *Biol. Reprod.*, **3**, 298–307.

Dandekar, P., Martin, M. and Glass, R. (1991) Maturation of immature oocytes by coculture with granulosa cells. *Fertil. Steril.*, **55**, 95–99.

Das, K., Stout, L.E., Hensleigh, H.C. *et al*. (1991) Direct positive effect of epidermal growth factor on the cytoplasmic maturation of mouse and human oocytes. *Fertil. Steril.*, **55**, 1000–1004.

Downs, S.M. (1989) Specificity of epidermal growth factor action on maturation of the murine oocyte and cumulus oophorus *in vitro*. *Biol. Reprod.*, **41**, 371–379.

Downs, S.M., Schroeder, A.C. and Eppig, J.J. (1986) Serum maintains the fertilizability of mouse oocytes matured *in vitro* by preventing the hardening of zona pellucida. *Gamete Res.*, **15**, 115–122.

Downs, S.M. and Mastropolo, A.M. (1997) Culture conditions affect meiotic regulation in cumulus-cell enclosed mouse oocytes. *Mol. Reprod. Dev.*, **46**, 551–566.

Dozortsev, D., De Sutter, P., Rybouchkin, A. and Dhont, M. (1996) Methodology of intracytoplasmic sperm injection in the human. *Assist. Reprod. Rev.*, **6**, 38–44.

Edwards, R.G. (1965a) Maturation *in vitro* of human oocytes. *Lancet*, **2**, 926.

Edwards, R.G. (1965b) Maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature*, **208**, 349–351.

Eppig, J., O'Brien, M. and Wigglesworth, K. (1996) Mammalian oocyte growth and development *in vitro*. *Mol. Reprod. Dev.*, **44**, 260–273.

Fukuda, Y., Ichikawa, M., Natio, K. and Toyoda, Y. (1990) Birth of normal calves resulting from bovine oocytes matured, fertilized and cultured with cumulus cells *in vitro* up to the blastocyst stage. *Biol. Reprod.*, **42**, 114–119.

Fukui, Y. (1990) Effect of follicle cells on the acrosome reaction, fertilization and developmental competence of bovine oocytes matured in vitro. *Mol. Reprod. Dev.*, **26**, 40–46.

Funahashi, H. and Day, B.N. (1993). Effects of different serum supplements in maturation medium on meiotic and cytoplasmic maturation of pig oocytes. *Theriogenology*, **39**, 965–973.

Gill, G.N. (1990) Regulation of EGF receptor expression and function. *Mol. Reprod. Dev.*, **27**, 46–53.

Gomez, E., Tarin, J.J. and Pellicer, A. (1993) Oocyte maturation in humans: the role of gonadotropins and growth factors. *Fertil. Steril.*, **60**, 40–46.

Haghinat, N. and Van Winkle, L.J. (1990) Developmental change in follicular cell-enhanced amino acid uptake into mouse oocytes that depends on intact gap junctions and transport system Gly. *J. Exp. Zool.*, **253**, 71–82.

Harper, K.M. and Brackett, B.G. (1993) Bovine blastocyst development after *in vitro* maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins. *Biol. Reprod.*, **48**, 409–416.

Hoffmann, G.E., Scott, R.T., Brzyski, R.G. and Jones, H.W. (1990) Immunoreactive epidermal growth factor concentrations in follicular fluid obtained from *in vitro* fertilization. *Fertil. Steril.*, **54**, 303–307.

Janssenswillen, C., Nagy, Z.P. and Van Steirteghem, A. (1995) Maturation of human cumulus-free germinal vesicle-stage oocytes to metaphase II by coculture with monolayer Vero cells. *Hum. Reprod.*, **10**, 375–376.

Kennedy, J.F. and Donahue, R.P. (1969) Human oocytes: maturation in chemically defined media. *Science*, **164**, 1292–1293.

Kito, S. and Bavister, B.D. (1997) Maturation of hamster oocytes under chemically defined conditions and sperm penetration through the zona pelucida. *Zygote*, **4**, 199–210.

Legge, M. (1995) Oocyte and zygote zona pellucida permeability to macromolecules. *J. Exp. Zool.*, **271**, 145–150.

Lonergan, P., Carolan, C., Van Langendonckt, A. *et al*. (1996) Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro*. *Biol. Reprod.*, **54**, 1420–1429.

Lorenzo, P.L., Rebollar, P.G., Illera, M.J. *et al*. (1996) Stimulatory effect of insulin-like growth factor I and epidermal growth factor on the maturation of rabbit oocytes *in vitro*. *J. Reprod. Fertil.*, **107**, 109–117.

Maruo, T., Ladines-Llave, C.A., Samoto, T. *et al*. (1993) Expression of

epidermal growth factor and its receptor in the human ovary during follicular growth and regression. *Endocrinology*, **132**, 924–931.

Moor, R.M. and Trounson, A.O. (1977) Hormonal and follicular factors affecting maturation of sheep oocytes *in vitro* and their subsequent developmental capacity. *J. Reprod. Fertil.*, **49**, 101–109.

Motta, P.M., Makabe, S., Naguro T. and Correr, S. (1994) Oocyte follicular cells association during development of human ovarian follicle. A study by high resolution scanning and transmission electron microscopy. *Arch. Histol. Cytol.*, **57**, 369–394.

Prins, G.S., Wagner, C., Weidel, L. *et al*. (1987) Gonadotropins augment maturation and fertilization of human immature oocytes cultured. *Fertil. Steril.*, **47**, 1035–1037.

Russell, J.B., Knezevich, K.M., Fabian, K.F. and Dickson, J.A. (1997). Unstimulated immature oocyte retrieval: early versus mid follicular endometrial priming. *Fertil. Steril.*, **67**, 616–620.

Schramm, R.D. and Bavister, B.D. (1996) Granulosa cells from follicle stimulating hormone-primed monkeys enhance the development competence of *in vitro* matured oocytes from non-stimulated rhesus monkeys. *Hum. Reprod.*, **11**, 1698–1702.

Sirard, M.A., Parrish, J.J., Ware, C.B. *et al*. (1988) The culture of bovine oocytes to obtain developmentally competent embryos. *Biol. Reprod.*, **39**, 546–552.

Staigmiller, R.B. and Moor, R.M. (1984) Effect of follicle cells on the maturation and development competence of ovine oocytes matured outside the follicle. *Gamete Res.*, **9**, 221.

Trounson, A.O., Bongso, A., Szell, A. *et al*. (1996) Maturation of human and bovine primary oocytes *in vitro* for fertilization and embryo production. *Singapore J. Obstet. Gynaecol.*, **27**, 78–84.

Trounson, A., Wood, C. and Kausche, A. (1994) In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertil. Steril.*, **62**, 353–362.

Vanderhyden, B.C. and Armstrong, D.T. (1989) Role of cumulus cells and serum on the in vitro maturation, fertilization and subsequent development of rat oocytes. *Biol. Reprod.*, **40**, 720–728.

Westergaard, L.G. and Andersen, C.Y. (1989) Epidermal growth factor (EGF) in human preovulatory follicles. *Hum. Reprod.*, **4**, 257–260.

Younis, A.I., Brackett, B.G. and Fayrer-Hosken, R.A. (1989) Influence of serum and hormones on bovine oocyte maturation and fertilization *in vitro*. *Gamete Res.*, **23**, 189–201.

Received on December 4, 1997; accepted on March 3, 1998