

Article

Effects of growth factors and granulosa cell co-culture on in-vitro maturation of oocytes



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Abstract

The maturation medium for in-vitro oocyte maturation is usually supplemented with serum. However, supplementation with serum from pregnant women adversely affects the outcome of in-vitro maturation. The purpose of the study was to assess if growth factors or granulosa cell co-culture could overcome the adverse effects of pregnant women's serum. The basal maturation medium consisted of TCM199, 75 mIU/ml human menopausal gonadotrophin (HMG), 0.2 mmol/l pyruvate, and 10% serum. The maturation medium for control 1 contained fertile women's serum. The maturation medium for control 2 contained pregnant women's serum. The maturation media for the study groups consisted of medium for control 2, with the addition of EGF, IGF-I, activin, TGF β or granulosa cell co-culture. Immature oocytes were obtained from FVB mice, and the experiment was repeated six times. After maturation, the oocytes were fertilized and cultured to blastocysts, and the cumulus cells were analysed for apoptosis. The maturation, fertilization and blastocyst rates of the control 2 group were significantly lower than those of control 1 group ($P < 0.05$). Addition of EGF, IGF-1, activin, TGF β or granulosa cell co-culture could not improve the outcome of in-vitro maturation. Cumulus cell proliferation was inhibited by pregnant women's serum. Apoptosis of cumulus cell was not related to in-vitro oocyte maturation and subsequent embryo development.

Keywords: apoptosis, co-culture, granulosa cell, growth factor, in-vitro maturation

Introduction

Recovery of immature oocytes followed by in-vitro maturation (IVM) and fertilization is a potential treatment for infertility. Compared with conventional IVF, IVM avoids the complications associated with ovarian stimulation and decreases patients' costs and suffering. Recent studies showed that IVM is a feasible alternative to traditional IVF (Chian *et al.*, 2004; Lin and Hwang, 2006).

In 1991, Cha *et al.* reported the first human birth resulting from IVM using immature oocytes obtained from an oophorectomy specimen (Cha *et al.*, 1991). In 1994, Trounson *et al.* reported the first birth from in-vitro matured oocytes in PCOS women (Trounson *et al.*, 1994). Immature oocytes can also be obtained

from pregnant women during Caesarean section. The oocytes can be matured *in vitro*, fertilized and frozen for future pregnancy if the women have infertility problems. Immature oocytes obtained from Caesarean section can also be used for oocyte donation. Without the need of ovarian stimulation and oocyte retrieval procedure, the women are more willing to be oocyte donors. Pregnancy resulting from immature oocytes obtained during Caesarean section has been reported (Hwang *et al.*, 1997).

Follicular maturation is continuous from 5 months of fetal life until menopause. Follicular growth continues even during pregnancy, although the process stops before ovulatory size

(Nelson and Greene, 1958). It has been shown in pregnant mares that the number of large follicles decreased in late pregnancy, but the number of small follicles was the same throughout pregnancy (Squires *et al.*, 1974). Low FSH concentrations resulting from negative feedback by increasing oestrogen concentrations impede further follicular development in late pregnancy. Even so, immature oocytes can be found in pregnant women (Hwang *et al.*, 1997, 2000; Hwu *et al.*, 1998).

However, the immature oocytes obtained during pregnancy seem to be of inferior quality, and pregnancies resulting from them are limited. In a previous study using immature oocytes obtained during Caesarean section, the maturation rate after 48 h was only 42.8% (Hwang *et al.*, 2000), which was lower than 74.2% in another study on women with polycystic ovary syndrome (PCOS) (Lin *et al.*, 2003). It has been demonstrated that supplementation of serum from pregnant women impaired oocyte maturation and fertilization (Lin *et al.*, 2008). The results provide indirect evidence of why immature oocytes from pregnant women do not perform as well as those from non-pregnant women.

Several growth factors, such as epidermal growth factor (EGF) (Das *et al.*, 1991; Gomez *et al.*, 1993; De La Fuente *et al.*, 1999), insulin-like growth factor-I (IGF-I) (Gomez *et al.*, 1993; Feng *et al.*, 1988; Lorenzo *et al.*, 1997; Pawshe *et al.*, 1998), activin (Alak *et al.*, 1998; Miro and Hillier, 1996), transforming growth factor- β (TGF β) (Feng *et al.*, 1988), and granulosa cell coculture (Schramm and Bavister, 1996) have been reported to improve oocyte maturation. The purpose of the study was to assess if these growth factors or granulosa cell co-culture could overcome the detrimental effects of serum from pregnant women.

During oocyte maturation, there is close interaction between oocyte and the surrounding cumulus cells. Cumulus cells support oocyte maturation and enhance cytoplasmic maturation, which is responsible for its capacity to undergo normal fertilization and subsequent embryonic development. Cumulus cells have been shown to undergo apoptosis during IVM (Ikeda *et al.*, 2003), and can protect oocytes against oxidative stress-induced apoptosis during IVM (Tatemoto *et al.*, 2000). Another purpose of the study was to see if the detrimental effects of pregnant women's serum were linked to apoptosis of cumulus cells.

Materials and methods

The chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless stated otherwise. The blood samples were obtained from six term pregnant women who were going to undergo scheduled Caesarean section. These women did not have any systemic diseases or complications associated with pregnancy. The blood was allowed to clot, and the serum was separated by centrifugation at 300 g for 10 min. The serum was heated at 56°C to inactivate complement, and was stored at -70°C until use in IVM.

For the control group, blood samples were obtained from six healthy and fertile women in their early follicular phase. The preparation of serum was the same as described above.

For the preparation of granulosa cells, granulosa cells were obtained from mature follicles during IVF and were frozen until

use. Two days before immature oocyte collection, granulosa cells at a density of 10^5 /ml were placed in the four-well dishes (Nunc, Copenhagen, Denmark) containing maturation medium [TCM 199, plus 75 mIU/ml human menopausal gonadotrophin (HMG; Pergonal; Serono, Geneva, Switzerland), 0.2 mmol/l pyruvate, and 10% fetal bovine serum (FBS)] and was incubated at 37°C in an atmosphere of 5% CO₂ in air. After 24 h, the unattached granulosa cells were washed away with maturation medium. The attached granulosa cells were cultured for one more day for co-culture with immature oocytes.

In-vitro maturation of oocytes

The mice used in the study were FVB/NJNarL mice (National Laboratory Animal Centre, Taipei, Taiwan). Immature oocytes were obtained from 3- to 5-week-old female mice, 48 h after intraperitoneal injection with 5 IU PMSG. Immature oocytes with cumulus cells were collected by teasing the ovarian follicles. Only cumulus-oocyte complexes (COC) that comprised oocytes with homogeneous cytoplasm and more than three layers of cumulus cells were used for the study. The COC were placed in groups in various maturation media. The basal maturation medium consisted of TCM199, 75 mIU/ml HMG (Merional; IBSA, Lamone, Switzerland), 0.2 mmol/l pyruvate, and 10% serum. The maturation medium for control 1 group contained fertile women's serum. The maturation medium for control 2 group contained pregnant women's serum. The maturation media for the study groups consisted of medium for control 2 group, with the addition of 10 and 20 ng/ml EGF, 50 and 100 ng/ml IGF-I, 100 and 200 ng/ml activin, 2.5 and 5 ng/ml TGF β , or granulosa cell co-culture separately in each group. Growth factors in two different concentrations were used to see if there were dose-dependent effects.

For IVM of oocytes, the COC were placed in the maturation media for 24 h. Only oocytes that displayed a distinct first polar body were classified as metaphase II (MII) and went through further fertilization and culture.

The spermatozoa were retrieved from the cauda epididymis of 5- to 7-week-old male mice. The spermatozoa were dispersed in HTF medium (Irvine Scientific, Santa Ana, CA, USA) plus 5 mg/ml FBS, and diluted to a concentration of 1×10^6 /ml.

The MII oocytes were incubated with spermatozoa for 4 h. After that, the oocytes were then washed to eliminate excess spermatozoa and then cultured overnight in a drop of potassium simplex optimized medium (mKSOM) (Speciality Media, Phillipsburg, NJ, USA) covered with mineral oil. On the next morning, the number of 2-cell embryos was counted, and the embryos were transferred to fresh droplets of mKSOM under mineral oil and cultured to blastocysts.

Fertilization was considered to be the percentage of 2-cell embryos 24 h after insemination. The maturation rates, fertilization rates, and embryo development were recorded.

Assessment of apoptosis

The cumulus cells that had undergone apoptosis were identified with the ApopTeg *In situ* Apoptosis Detection Kit (Chemicon, Catalog no. S7100). After 24 h of culture, the COC that were

going to be analysed for apoptosis were cultured in 80 IU/ml hyaluronidase for 30 s, and the cumulus cells were separated by repeated pipetting. The cumulus cells were collected in a test tube (Falcon, Franklin Lakes, NJ, USA) containing 2 ml of PBS and centrifuged twice at 300 *g* for 10 min. The pellet with the cumulus cells was pipetted onto silanized glass slides and a thin smear was prepared. The cells were fixed by immersing slides in a Coplin jar containing 1% paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature. The presence of DNA fragments was detected by the terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labelling (TUNEL) assay. The ApopTeg kit labels the free 3'-OH DNA termini with chemically labelled and unlabelled nucleotides. The nucleotides contained in the reaction buffer are added enzymatically to the DNA by terminal deoxynucleotidyl transferase (TdT). TdT catalyses a template-independent addition of nucleotide triphosphates to the 3'-OH ends of double- or single-stranded DNA. The incorporated nucleotides from an oligomer composed of digoxigenin are allowed to bind an anti-digoxigenin antibody that is conjugated to peroxidase. The localized peroxidase enzyme then catalytically generates an intense signal from chromogenic substrates. The cells were observed with a magnification of $\times 400$ and bright-field optics (Nikon Diaphot, Tokyo, Japan). About 200 cumulus cells were observed for each treatment and the percentage of cells with apoptotic nuclei was calculated as apoptotic index. The slides were observed by the same observer twice. The intra-observer variation was less than 2%.

Differential staining of blastocysts

For the differential staining of trophectoderm (TE) and inner cell mass (ICM), blastocysts were incubated in 0.05% pronase solution in mKSOM for 5 min at 37°C to remove zona pellucida. The blastocysts were washed in mKSOM and incubated in 0.5% 2,4,6-trinitrobenzene sulphonic acid solution for 10 min at 4°C in the dark, followed by a wash in mKSOM. The blastocysts were incubated at room temperature for 40 min in the dark in stain solution, which contained 1 $\mu\text{g/ml}$ bisbenzimidazole (Hoechst

33258) and 1 $\mu\text{g/ml}$ propidium iodide in mKSOM. The stained blastocysts were washed again in mKSOM before fixation in 4% formalin solution. They were then stored in the dark at 4°C until observation. The blastocysts were observed under fluorescence microscope equipped with a UV filter. The nuclei of ICM appeared blue and the nuclei of TE appeared pink.

Experiment design

The experiment was repeated six times. In each replica, serum from different pregnant and non-pregnant women was used. Forty COC were allocated in each group (control 1, control 2, EGF, $\times 2$ EGF, IGF-1, $\times 2$ IGF-1, activin, $\times 2$ activin, TGF β , $\times 2$ TGF β , and granulosa cell co-culture). In each group, 20 COC were matured *in vitro*, fertilized and cultured to blastocysts. Another 20 COC were matured *in vitro*, and were analysed for cumulus cell apoptosis.

Statistical analysis

Comparison of percentages was performed using chi-squared test or Fisher's exact test. Blastocyst cell counts were compared by independent *t*-test. $P < 0.05$ was considered statistically significant. Analyses were carried out using the Statistics Package for Social Sciences (SPSS), version 14.0 (SPSS Inc., Chicago, IL, USA).

Results

The outcomes of IVM are shown in **Table 1**. Compared with the standard maturation medium (control 1), supplementation with pregnant women's serum (control 2) decreased oocyte maturation rate, fertilization rate and blastocyst development. Addition of different growth factors, including EGF, IGF-1, activin and TGF β , did not improve maturation rate, fertilization rate, or embryo development, even if the concentrations were doubled. Co-culture with granulosa cells increased oocyte maturation and embryo development slightly, but not significantly.

Table 1. Outcome of in-vitro maturation of oocytes ($n = 120$) in different media.

Medium	MII oocytes	Fertilization rate	Blastocyst rate	Apoptosis index
Control 1	83.3 ^a	68.0 ^a	29.4 ^a	31.7
Control 2	65.0	51.2	10.0	32.8
EGF	65.0	52.6	7.3	24.6 ^a
$\times 2$ EGF	66.7	56.3	6.7	25.1 ^a
IGF-I	64.2	54.5	7.1	40.3 ^a
$\times 2$ IGF-I	65.0	57.0	8.9	43.7 ^a
Activin	65.8	55.7	11.4	53.4 ^a
$\times 2$ Activin	66.7	53.1	11.6	54.4 ^a
TGF β	63.3	50.0	10.5	50.7 ^a
$\times 2$ TGF β	63.3	52.6	10.0	51.3 ^a
Granulosa cell co-culture	78.3	54.3	19.6	29.4

Values are percentages of the 120 oocytes matured in each medium.

Control 1 = standard maturation medium containing fertile women's serum. Control 2 = standard maturation medium containing pregnant women's serum. The maturation media for the study groups consisted of medium for control 2 group, with the addition of 10 ng/ml EGF, 20 ng/ml EGF, 50 ng/ml IGF-I, 100 ng/ml IGF-I, 100 ng/ml activin, 200 ng/ml activin, 2.5 ng/ml TGF β and 5 ng/ml TGF β .

^a $P < 0.05$ compared with control 2.

After 24 h of IVM, the cumulus cells in the media containing pregnant women's serum were much fewer and less expanded, even if various growth factors were added. Cumulus cell apoptosis was not related to in-vitro oocyte maturation and subsequent embryo development. Although pregnant women's serum impaired IVM, the apoptosis indexes of cumulus cells between control 1 and control 2 were similar. Compared with control 2, addition of EGF decreased cumulus cell apoptosis. On the contrary, addition of IGF-I, activin and TGF β increased apoptosis.

The cell counts of blastocysts are shown in **Table 2**. Although fewer blastocysts were obtained in control 2 compared with control 1, total cell numbers, and cell numbers of TE and ICM were similar between control 1 and control 2. Addition of growth factors or granulosa cell co-culture did not significantly change the cell numbers of ICM and TE.

Discussion

The present study confirms previously published results (Lin *et al.*, 2008) showing that supplementation of pregnant women's serum impaired oocyte maturation, fertilization and development to blastocysts. Supplementation of various growth factors and granulosa cell co-culture could not reverse the adverse effects, even if the concentrations of growth factors were doubled. The adverse effects of pregnant women's serum may be through inhibition of cumulus cell proliferation.

The maturation medium for human IVM is usually supplemented with various protein sources, such as fetal bovine serum (Tounson *et al.*, 1994; Chian *et al.*, 2000), fetal cord serum (Cha *et al.*, 1991), or maternal serum (Mikkelsen *et al.*, 1999; Lin *et al.*, 2003). It is believed that serum contains albumin and other unknown constituents that are helpful for oocyte maturation. Despite different protein sources used, all of the studies gave comparable results.

However, in a previous study (Lin *et al.*, 2008), it was shown that oocyte maturation was impaired with the addition of pregnant women's serum. There was a trend towards decreasing maturation and fertilization rates with increasing concentration of women's serum. The detrimental effects of the pregnant women's serum were at least in part due to the hormonal changes during pregnancy.

The purpose of the study was to evaluate if addition of various growth factors or granulosa cell co-culture could overcome the detrimental effects of the pregnant women's serum. Unfortunately, the present study showed that various growth factors could not reverse the detrimental effects of pregnant women's serum. Granulosa cell co-culture slightly improved the maturation rate, but the development to blastocysts was still impaired.

Preovulatory LH surge induces not only the oocyte to resume meiosis but also the cumulus cells to multiply and expand. In the study, it was found that after 24 h of IVM, the cumulus cells in the media containing pregnant women's serum were remarkably fewer than the cumulus cells in the standard maturation medium, even if growth factors were added or co-cultured with granulosa cells. This indicates that pregnant women's serum inhibits cumulus cell proliferation.

Many studies have shown that EGF is beneficial to IVM. EGF can stimulate cumulus cell expansion (Longergan *et al.*, 1996), oocyte maturation (Das *et al.*, 1991; Gomez *et al.*, 1993; De La Fuente *et al.*, 1999), and blastocyst development (Longergan *et al.*, 1996). The effects may be related to increased H1 kinase and MAP (mitogen-activated protein) kinase activities in oocytes mediated by cumulus cells (Sakaguchi *et al.*, 2002). The beneficial effects of EGF on IVM have been demonstrated in several species, including mouse (Das *et al.*, 1991; De La Fuente *et al.*, 1999), cow (Longergan *et al.*, 1996; Oyamada *et al.*, 2004), pig (Ding and Foxcroft 1994) and human (Gomez *et al.*, 1993; Goud *et al.*, 1998). The effects are dose dependent. Its positive effect on oocyte maturation was

Table 2. Comparison of total cell numbers and cell numbers of TE and ICM of blastocysts derived from different media.

Medium	Blastocyst cells	TE cells	ICM cells
Control 1	47.7 $\pm$ 9.5	32.5 $\pm$ 6.7	15.3 $\pm$ 4.1
Control 2	45.2 $\pm$ 7.8	33.6 $\pm$ 7.2	11.6 $\pm$ 3.7
EGF	48.5 $\pm$ 8.3	32.9 $\pm$ 5.9	15.6 $\pm$ 3.9
$\times 2$ EGF	49.5 $\pm$ 9.1	34.2 $\pm$ 6.2	15.3 $\pm$ 4.0
IGF-I	47.5 $\pm$ 8.4	33.5 $\pm$ 6.8	14.0 $\pm$ 4.2
$\times 2$ IGF-I	46.8 $\pm$ 8.3	33.1 $\pm$ 6.0	13.7 $\pm$ 3.8
Activin	45.9 $\pm$ 9.0	34.1 $\pm$ 5.8	11.8 $\pm$ 4.1
$\times 2$ activin	47.1 $\pm$ 8.5	34.9 $\pm$ 6.1	12.2 $\pm$ 4.0
TGF β	48.1 $\pm$ 9.2	35.0 $\pm$ 5.8	13.1 $\pm$ 3.5
$\times 2$ TGF β	47.9 $\pm$ 8.8	34.1 $\pm$ 6.4	13.8 $\pm$ 4.3
Granulosa cell co-culture	48.3 $\pm$ 8.8	35.1 $\pm$ 6.9	13.2 $\pm$ 4.7

Values are mean $\pm$ SD.

Control 1 = standard maturation medium containing fertile women's serum. Control 2 = standard maturation medium containing pregnant women's serum. The maturation media for the study groups consisted of medium for control 2 group, with the addition of 10 ng/ml EGF, 20 ng/ml EGF, 50 ng/ml IGF-I, 100 ng/ml IGF-I, 100 ng/ml activin, 200 ng/ml activin, 2.5 ng/ml TGF β and 5 ng/ml TGF β .

borderline significant at a physiological concentration of 2 ng/ml (Westergaard and Andersen, 1989), but more evident at 10 ng/ml (Gomez *et al.*, 1993). However, the present study showed that EGF could not overcome the detrimental effects of pregnant women's serum, even at 20 ng/ml.

Other growth factors have been shown to be beneficial to IVM. IGF-I has been demonstrated to stimulate oocyte maturation (Feng *et al.*, 1988; Gomez *et al.*, 1993; Lorenzo *et al.*, 1997; Pawshe *et al.*, 1998) and embryo development (O'Neill, 1997; Lighten *et al.*, 1998; Pawshe *et al.*, 1998). Activin has been shown to enhance oocyte maturation (Miro and Hillier, 1996; Alak *et al.*, 1998) and increase development to blastocysts (Silva and Knight, 1998). TGF β also stimulates oocyte maturation in the rat (Feng *et al.*, 1988).

However, in the present study, it was found that these growth factors could not improve IVM, even at higher concentrations than reported in the literature. Similarly, Brzyski showed that EGF had no significant effect on IVM of baboon oocytes retrieved at Caesarean section (Brzyski *et al.*, 1999). The cumulus cells play an important role in the oocyte maturation process. It is probably mediated through the gap junction cytoplasmic connections to the ooplasm (Carabatsos *et al.*, 2000), or by the cumulus cells exerting a modulatory effect on the microenvironment surrounding the immature oocytes (Byskov *et al.*, 1997). The beneficial effects of growth factors have been shown to act through the cumulus cells (Downs *et al.*, 1988; Feng *et al.*, 1988; Lorenzo *et al.*, 1996, 1997; Sakaguchi *et al.*, 2002). The present study showed that pregnant women's serum inhibited cumulus cell proliferation, which may explain why the growth factors could not reverse its adverse effects on IVM. In addition, serum contains binding proteins such as α_2 -macroglobulin, which has been reported to bind activin (Vaughan and Vale, 1993) and TGF β (Feige *et al.*, 1996), may block the effects of growth factors on IVM.

Co-culture has been used in assisted reproduction to improve embryonic development (Plachot *et al.*, 1993). Although Trounson *et al.* (1994) showed in a few cases that co-culture with mature granulosa cells did not improve the maturation and fertilization rates, Schramm and Bavister (1996) demonstrated that co-culture with FSH-primed granulosa cells enhanced the development to morulae and blastocysts of in-vitro matured monkey oocytes. Similarly, Hwu *et al.* (1998) showed that ampullary cell co-culture enhanced blastocyst development of in-vitro matured human oocytes. It is still unclear how co-culture supports IVM, but it is likely that co-culture cells secrete growth factors or other constituents that enhance oocyte maturation, rather than removing toxic products from the culture medium (Schramm and Bavister, 1996). The present study showed that granulosa cell co-culture could not overcome the adverse effects of pregnant women's serum either. The beneficial effects of co-culture are probably through the cumulus cells too, thus it cannot exert its effects when there are sparse cumulus cells.

Although blastocyst development was impaired by the addition of pregnant women's serum, blastocyst quality was unaffected. This is reflected by similar total blastocyst cell numbers and cell numbers of TE and ICM.

Cumulus cell apoptosis does not occur during in-vitro oocyte maturation (Szoltyś *et al.*, 2000). However, ovarian

stimulation induces cumulus cell apoptosis, which has been reported to be inversely related to oocyte maturity and IVF outcome (Nakahara *et al.*, 1997; Host *et al.*, 2000). Ikeda *et al.* also demonstrated that cumulus cells in bovine COC underwent apoptosis during IVM in a time dependent fashion, and the degree of apoptosis was related to the developmental competence of the enclosed oocytes (Ikeda *et al.*, 2003). Since cumulus cells provide nutrients and signals that regulate oocyte growth and maturation (Tanghe *et al.*, 2002), it was postulated that increased cumulus cell apoptosis occurs in the presence of pregnant women's serum. In the present study, it was found that after 24 h of maturation *in vitro*, the cumulus cells were sparse in the media supplemented with pregnant woman's serum, but the apoptosis indexes between control 1 and control 2 groups were similar. The effects of growth factors on cumulus cell apoptosis were inconsistent. Compared with control 2, addition of EGF decreased apoptosis index, but addition of IGF-I, activin, and TGF β increased apoptosis index. The apoptosis index was not associated with the maturation rate and blastocyst development. This indicates that cumulus cell apoptosis plays little role, if any, in IVM in the presence of sparse cumulus cells.

In conclusion, this study showed that supplementation of culture medium with pregnant women's serum decreased in-vitro oocyte maturation and blastocyst development. Addition of growth factors and granulosa cell co-culture could not reverse its adverse effects. The adverse effects of pregnant woman's serum might be via the cumulus cells.

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