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Effect of recombinant human gonadotrophins on human, bovine and murine oocyte meiosis, fertilization and embryonic development *in vitro*

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The response of murine, bovine and human oocytes to pure recombinant preparations of human follicle stimulating hormone (rFSH) and luteinizing hormone (rLH) for meiotic maturation and subsequent developmental competence *in vitro* were examined in the present experiments. Maturation of immature bovine oocytes to the metaphase II stage was significantly increased by the addition of 1 IU/ml of rFSH in combination with either 1 IU/ml rLH or 10 IU/ml rLH. Similarly, embryonic development to the blastocyst stage was improved in bovine oocytes treated with a 1:10 combination of rFSH:rLH. However, no significant difference was observed in the number of inner cell mass or trophectoderm cells of the resulting blastocysts. Although the increased maturation to metaphase II was not significant, human embryonic developmental competence was improved by maturing oocytes in the presence of a 1:10 ratio of rFSH:rLH as only those oocytes exposed to a 1:10 ratio of rFSH: rLH during maturation showed normal cleavage patterns beyond day 2. In addition, 1 IU/ml rFSH and 1 IU/ml rLH increased the expression of oocyte proteins in human oocytes. The inclusion of recombinant gonadotrophins, either singly or in combination, had no significant effect on the maturation, fertilization or embryonic development of in-vitro matured mouse oocytes. These data provide support for the responsiveness of human and bovine oocytes to gonadotrophins *in vitro* and the need to consider variations in the relative concentrations for optimization of oocyte developmental competence.

Key words: embryonic development/immature oocytes/in-vitro maturation/oocyte proteins/recombinant gonadotrophins

Introduction

Human immature oocytes are capable of undergoing meiotic maturation and fertilization *in vitro* (Cha *et al.*, 1991; Trounson *et al.*, 1994; Barnes *et al.*, 1996; Wynn *et al.*, 1998). However, subsequent preimplantation development is abnormal as human embryos display both retarded cleavage and blockage of development (Trounson *et al.*, 1996, 1998).

The hypophysial gonadotrophins FSH and LH have an important role in the regulation of follicular growth (Abir *et al.*, 1997) and oocyte meiotic maturation (Singh *et al.*, 1993). *In vitro*, non-recombinant FSH and LH have been shown to enhance fertilization and embryo development of immature bovine oocytes (Younis *et al.*, 1989; Zuelke and Brackett, 1990). Furthermore, the supplementation of maturation medium with elevated concentrations of non-recombinant LH has been shown to augment further the maturation, fertilization and developmental capabilities of in-vitro matured oocytes (Brackett *et al.*, 1989; Keefer *et al.*, 1993).

Recombinant gonadotrophins have been demonstrated to stimulate ovulation, maturation and steroidogenesis in rats (Galway *et al.*, 1990; Törnell *et al.*, 1995). However, studies utilizing recombinant gonadotrophins for the in-vitro maturation of immature oocytes are limited largely to studies in rodent oocytes (Törnell *et al.*, 1995; Byskov *et al.*, 1997; Cortvrindt *et al.*, 1998) and the effect of recombinant gonadotrophins on embryonic development is largely unexplored.

The present studies examined the response of the isolated cumulus oocyte complexes of three species, the mouse, cow and human, to maturation *in vitro* without gonadotrophins or maturation with pure (recombinant) human FSH (rFSH) and LH (rLH) at ratios of 1:1 and 1:10. The latter ratio was chosen to mimic gonadotrophin ratios when the LH surge occurs at the time of ovulation and maturation *in vivo*. The normality of oocyte maturation was assessed by progression to metaphase II and the developmental competence of fertilized oocytes *in vitro*.

Materials and methods

The Epworth Hospital Research and Ethics Committee approved the studies involving human oocyte maturation. Human oocytes were collected either as part of an IVF treatment cycle or following therapeutic ovarian drilling for resumption of menstrual cyclicity. In all cases informed consent was obtained from all patients who donated human oocytes for this research. Human embryos, which were generated following IVF, were cultured for 5 days *in vitro*. One to three embryos continuing to cleave over a 5 day culture period were transferred back to the patient as previously described (Trounson *et al.*, 1994).

Ethics approval for animal studies was granted by the Monash University Animal Ethics Committee.

Lyophilized rFSH (Gonal-F; Serono, Geneva, Switzerland) and rLH (LADI; Serono, Geneva, Switzerland) were reconstituted in

sterile water and added to the maturation medium prior to oocyte culture. rFSH and rLH have been demonstrated to display high biopotencies in rodent cells (Hakola *et al.*, 1998) and have been previously successfully used to induce meiotic maturation and steroidogenesis in rodent oocytes and follicles (Törnell *et al.*, 1995; Cortvrindt *et al.*, 1998). Similarly, human follicular cells have been shown to be highly responsive to rFSH and rLH (Bergh *et al.*, 1997) and rFSH and rLH have been successfully used for the maturation of bovine oocytes *in vitro* (Chanson *et al.*, 1997). Therefore, due to the efficacy of rFSH and rLH in human, rodent and bovine cells, human recombinant gonadotrophins were used in this study. The concentrations of gonadotrophins used in this study were based on concentrations previously used for mouse and bovine in-vitro maturation and those considered appropriate for use in human oocyte culture (Chanson *et al.*, 1997). Similarly, the oocyte and embryo culture conditions used in this study have been shown to be most effective for supporting embryonic development (Gardner *et al.*, 1994; Anderiesz *et al.*, 1995; Fong *et al.*, 1997; Jones *et al.*, 1998).

Retrieval and in vitro maturation of human oocytes

Human oocytes were retrieved from women who had not received any exogenous gonadotrophin stimulation prior to oocyte retrieval. Immature human oocytes were recovered by follicular aspiration and isolated by techniques previously described (Trounson *et al.*, 1994).

Following isolation, groups of up to four human cumulus oocyte complexes (COCs) were matured for 48 h in 1 ml Tissue Culture Medium 199 (TCM 199; Sigma, St Louis, MO, USA) supplemented with 2 mg/ml human serum albumin (HSA; Irvine Scientific, Santa Ana, CA, USA) and either 1 IU/ml rFSH for 48 h (FSH group) or 1 IU/ml rFSH for 24 h and then 1 IU/ml rFSH in combination with 10 IU/ml rLH (Serono) for the second 24 h (1:10 group), or no gonadotrophins for the duration of the 48 h of culture (no hormone group). Oocytes were matured in sterile 5 ml plastic tubes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 37°C in humidified 5% CO_2 in air.

Retrieval and in-vitro maturation of bovine oocytes

Bovine ovaries were obtained from 9–15 month old cows at the abattoir. The ovaries were heterogeneous in size, ranging from 3 to 6 cm in length and ~60% contained no corpora lutea. COCs were recovered by aspirating ovarian follicles >2 mm in diameter with a 19 gauge$\times\frac{3}{4}$ inch needle. The majority of the follicles aspirated were <10 mm in diameter. Follicular fluid was pooled in 10 ml tubes (Falcon) and the COCs were sedimented by gravity for 30 min. The COCs were collected and washed twice in TCM 199-Hepes (Sigma) with 4 mg/ml bovine serum albumin (BSA; Pentex crystallized, Miles, Kankakee, IL, USA) and once in TCM 199 with 10% fetal calf serum [FCS; Commonwealth Serum Laboratories (CSL), Melbourne, VIC, Australia]. Up to 50 COCs were randomly selected and placed into organ culture dishes (Falcon) in 1 ml of TCM 199 supplemented with 10% FCS and either 1 IU/ml rFSH (FSH group) or 1 IU/ml rLH (LH group) or 1 IU/ml rFSH in combination with 1 IU/ml rLH (1:1 group) or 1 IU/ml rFSH in combination with 10 IU/ml rLH (1:10 group) or no gonadotrophins (no hormones group). The cultures were maintained for 24 h at 39°C in humidified 5% CO_2 in air and the maturation medium was supplemented by the gonadotrophins for the duration of the 24 h maturation interval.

Retrieval and in-vitro maturation of murine oocytes

Four to 6 week old female F1 hybrid mice (C57BL/6J WEHI female×CBA/CaH WEHI male) were given an i.p. injection of 5 IU pregnant mares' serum gonadotrophin (PMSG; Folligon; Intervet, NSW, Australia) to induce follicular recruitment and increase the number of oocytes collected. Forty-eight hours after PMSG injection, animals were killed by cervical dislocation, the ovaries dissected and placed in M2 culture medium (Quinn *et al.*, 1982) containing 4 mg/ml BSA. COCs were released from the follicles using a 16-gauge needle and collected into M2 medium. Groups of up to 15 COCs were cultured in 35 mm tissue culture dishes (Nunclon, Roskilde, Denmark) in 20 µl drops of Eagle's minimal essential medium – alpha modification (α-EMEM; Sigma) supplemented with 10% FCS and either 0.2 IU/ml rFSH (FSH group) or 0.2 IU/ml rLH (LH group) or 0.2 IU/ml rFSH in combination with 0.2 IU/ml rLH (1:1 group) or 0.2 IU/ml rFSH in combination with 2.0 IU/ml rLH (1:10 group) or no gonadotrophins (no hormones). The cultures were maintained for 18 h at 37°C in humidified 5% CO_2 in air. Maturation medium was supplemented with all the gonadotrophins for the duration of the 18 h maturation interval.

IVF of human oocytes

When compared to conventional insemination techniques, intracytoplasmic sperm injection (ICSI) results in a higher rate of fertilization in human in-vitro matured oocytes (Barnes *et al.*, 1995; Nagy *et al.*, 1996). Thus, in the present study, human oocytes were inseminated using ICSI according to previously described methods (Palermo *et al.*, 1992). Briefly, freshly ejaculated semen samples were prepared according to conventional swim up procedures (Mahadevan and Trounson, 1984). The cumulus cells were removed by enzymatic digestion with hyaluronidase (Hyase®, Scandinavian IVF Science AB, Gothenburg, Sweden) and the coronal cells were dispersed by gentle pipetting with a finely pulled Pasteur pipette. The denuded oocytes were washed in modified human tubal fluid medium (IVF-50; Scandinavian IVF Science AB), classified according to nuclear maturity by microscopic evaluation and then placed in fresh IVF-50 medium until the time of injection. Following ICSI, oocytes were transferred to 100 µl drops of IVF-50 medium and covered in light paraffin oil (Ovoil; Scandinavian IVF Science AB). Successful fertilization was assessed by the appearance of two pronuclei and the extrusion of the second polar body, 14–16 h following ICSI.

IVF of bovine oocytes

Frozen–thawed semen from a single bull was used for the fertilization of all oocytes. The thawed spermatozoa were layered on a discontinuous Percoll (Sigma) – Sperm Talp (Parish *et al.*, 1985) gradient (50, 70, 90%) supplemented with 6 mg/ml BSA and centrifuged at 600 *g* for 20 min. The sperm pellet was removed and washed by centrifugation for 5 min at 300 *g*. The sperm pellet was then resuspended in 5 ml of fertilization Talp medium (Bavister and Yanagimachi, 1977) supplemented with 5 µg/ml heparin (Sigma) and 6 mg/ml BSA. Groups of ten oocytes were inseminated in 50 µl drops of Fertilization Talp medium containing 2×10^2 spermatozoa/ml under light white mineral oil (Sigma). Oocytes were inseminated for ~20 h at 39°C in humidified 5% CO_2 in air.

IVF of mouse oocytes

Mouse oocytes were fertilized *in vitro* using spermatozoa retrieved from the cauda epididymides of two mature F1 (C57BL/6J WEHI female×CBA/CaH WEHI male) mice. The details and methods of mouse IVF have been previously described (Anderiesz and Trounson, 1995). Following insemination, oocytes were washed in M16 (Quinn *et al.*, 1982) supplemented with 4 mg/ml BSA and cultured for a further 4 h. Successful fertilization was assessed by the appearance of 2 pronuclei and the extrusion of the second polar body 8–10 h following insemination.

Human embryonic development following IVF

Pronuclear human zygotes were cultured in 100 µl drops of IVF-50 medium (Scandinavian IVF Sciences AB, Gothenburg, Sweden) at 37°C in humidified 5% CO_2 in air for 3 days following ICSI. On day 3, the oocytes were washed and transferred to 100 µl drops of Hatch-50 medium (Scandinavian IVF Science AB) and the embryos were cultured for a further 2 days at 37°C in a humidified atmosphere of 5% CO_2 in air. On each day, embryonic cleavage was recorded. Embryos that were cleavage arrested prior to day 5 were discarded, whereas embryos that continued to cleave up to day 5 were transferred to the patient.

Bovine embryonic development following IVF

Following insemination, oocytes were vortexed briefly to remove cumulus cells and washed in synthetic oviductal fluid (SOF) medium supplemented with 1% v/v non-essential amino acids (NE-AA; ICN, Aurora, Ohio, USA) and 8 mg/ml BSA. Groups of 50 presumptive zygotes were transferred to 4-well culture dishes (Nunclon) containing 500 µl of SOF with NE-AA (ICN) and 8 mg/ml BSA covered with 300 µl light white mineral oil and cultured for 72 h at 39°C in 7% O_2, 5% CO_2, 88% N_2. After 72 h, embryos were transferred to 500 µl of SOF medium supplemented with 8 mg/ml BSA and 2% (v/v) 20 amino acids (20AA; ICN), covered with 300 µl light white mineral oil and then cultured until day 7 at 39°C in 7% O_2, 5% CO_2 and 88% N_2. Development to blastocysts was recorded on day 7 after insemination.

Mouse embryonic development following IVF

Following insemination, adherent cumulus cells were removed from oocytes mechanically by gentle micropipetting with a fine drawn glass pipette. Mouse zygotes were cultured for 96 h in 20 µl drops of M16 supplemented with 4 mg/ml BSA under light white mineral oil in humidified 5% CO_2 in air (Anderiesz and Trounson, 1995). Development to blastocysts was recorded on day 4 after insemination.

Differential labelling of trophectoderm and inner cell mass cells

The number of trophectoderm (TE) and inner cell mass (ICM) cells were counted in bovine embryos developing to the blastocyst stage on day 7. The technique used was a modification of protocols previously described (Handyside and Hunter, 1984) and involved the differential labelling of TE and ICM cells with the flurochromes propidium iodide (Sigma) and bisbenzimide (Hoechst 33342; Sigma). Briefly, the zonae pellucidae of the blastocysts were dissolved by incubation in 0.5% Pronase (Sigma) for 5 min at 39°C. The embryos were then washed in M2 culture medium and incubated in rabbit anti-bovine serum (ICN) for 30 min and then placed in a 1:1 dilution of guinea pig complement (ICN) and 20 µg/ml propidium iodide for 20 min at 39°C. The embryos were finally placed in 25 µg/ml bisbenzimide in ethanol (British Drug House, Kilsyth, VIC, Australia) and maintained at 4°C overnight. Counting of cells was performed on a Zeiss fluorescent microscope after fixing the embryos on a glass slide with glycerol (Sigma).

Investigation of oocyte proteins following in-vitro maturation

Human, bovine and mouse oocyte proteins were separated by one-dimensional SDS–PAGE. Groups of 10 murine, three bovine and single human metaphase II oocytes were enzymatically denuded of cumulus cells in 40 IU hyaluronidase (Sigma) and placed as groups or individual cells in 10 µl sample buffer (Laemmli, 1970) and boiled for 3 min to lyse and solubilize the oocyte and its proteins. To avoid dilution and loss of sample, the lysis and solubilization of the oocytes was carried out in a single tube containing 10 µl of Laemmli sample buffer and the entire 10 µl of sample was subsequently loaded on the gel. The proteins were separated on a sodium dodecyl sulphate 12% polyacrylamide gel electrophoresis (SDS–PAGE) in accordance with previously described methods (Laemmli, 1970). The gels were run at 150 V for 1 h on a mini Protean II electrophoresis system (Bio-Rad, Hercules, CA, USA). Following electrophoresis the gels were silver stained (Heukeshaven and Dernick, 1988) to visualize proteins and air-dried overnight between two sheets of acetate paper (Promega, Annandale, NSW, Australia) at room temperature. Three to five replicates of human, bovine and murine oocytes were separated by electrophoresis with additional control medium and cumulus cell controls.

Statistics

Replicate data were analysed for homogeneity and results are expressed as mean ± SEM. Proportionate data were converted to a percentage and meiotic maturation, fertilization, development to blastocyst and cell number were analysed using analysis of variance (ANOVA) and Tukey–Kramer post tests for multiple comparisons. $P < 0.05$ was accepted as the minimum level of statistical significance.

Results

In-vitro maturation of oocytes

The addition of human recombinant gonadotrophins increased the maturation of human oocytes to metaphase II (Figure 1a). Specifically, the addition of rFSH alone or a 1:10 ratio of rFSH to rLH increased the maturation of human oocytes by 29% ($n = 22$) and 39% ($n = 33$) respectively compared to the maturation of oocytes with no hormones (Figure 1a). However, these differences were not statistically significant.

Inclusion of recombinant gonadotrophins increased the percentage of bovine oocytes maturing to metaphase II (Figure 1b). Moreover, significantly fewer ($P < 0.01$) bovine oocytes matured in medium containing no hormones than in medium supplemented with a 1:1 or 1:10 combination of rFSH and rLH (Figure 1b).

The inclusion of either rFSH or rLH, in the mouse maturation medium, was of no detectable benefit to meiotic maturation. In all experimental groups, consistently high proportions of mouse oocytes matured to metaphase II (Table I).

IVF

Human oocyte fertilization was higher in oocytes treated with rFSH (89%, $n = 10$) and 1:10 ratio rFSH and rLH (87%, $n = 24$) than human oocytes matured with no hormones (67%, $n = 6$). However, the differences were not statistically significant.

The direct assessment of fertilization in bovine oocytes is difficult due to the high lipoprotein content of the cytoplasm. Therefore, the cleavage of bovine oocytes was used as an indirect and approximate assessment of fertilization success. Embryonic cleavage was not significantly different ($P = 0.4895$) in the no hormone group (60%, $n = 173$), the FSH group (63%, $n = 169$), the LH group (58%, $n = 180$), the 1:1 group (72%, $n = 193$) or the 1:10 group (71%, $n = 163$), demonstrating that the cleavage of bovine embryos was not affected by the addition of gonadotrophins either singly or in combination.

A statistically similar ($P = 0.935$) and comparable percent-

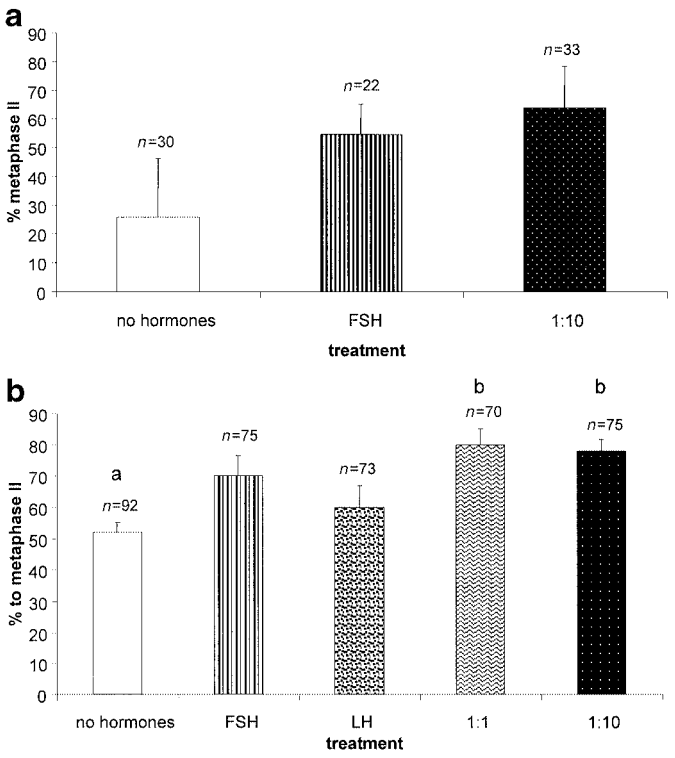

Figure 1. In-vitro maturation of oocytes to metaphase II. (**a**) Human: effect of no hormones, human rFHS (1 IU/ml) alone or rFHS (1 IU/ml) in combination with rLH (10 IU/ml on in-vitro maturation of human cumulus enclosed oocytes. Results are expressed as mean ± SEM and *n* represents the total number of oocytes from several replicates. (**b**) Bovine: effect of no hormones, rFSH (1 IU/ml) alone, rLH (1 IU/ml) alone, rFSH (1 IU/ml) in combination with rLH (1 IU/ml) or rFSH (1 IU/ml) in combination with rLH (10 IU/ml) on the in-vitro maturation of cumulus enclosed oocytes. Results are expressed as mean ± SEM and *n* represents the total number of oocytes from several replicates. Bars labelled a and b are significantly different at $P < 0.01$.

Table I. Maturation, fertilization and embryonic development of murine oocytes

Treatment	Oocytes matured (%)	Oocytes fertilized (%)	Zygotes developed to blastocyst (%)
No hormones	92 ± 5 (n = 95)	63 ± 11 (n = 55)	18 ± 11 (n = 34)
FSH	93 ± 5 (n = 90)	67 ± 6 (n = 73)	19 ± 3 (n = 48)
LH	93 ± 6 (n = 66)	77 ± 7 (n = 60)	16 ± 4 (n = 37)
1 FSH:1 LH	89 ± 7 (n = 112)	62 ± 15 (n = 49)	27 ± 9 (n = 31)
1 FSH:10 LH	94 ± 3 (n = 120)	69 ± 8 (n = 72)	28 ± 8 (n = 45)

age of mouse oocytes fertilized *in vitro* in all treatment groups (Table I).

Embryonic development

Cleavage of human embryos was recorded for 5 days *in vitro*. Figure 2 illustrates human embryonic development expressed as the expected embryo cell number on the respective day of culture. Interestingly, only human oocytes matured in the presence of a 1:10 ratio of rFSH and rLH produced embryos that cleaved normally beyond day 2. A total of seven human

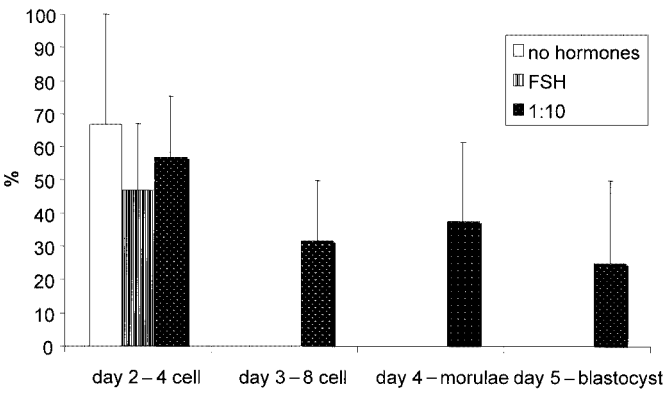

Figure 2. Cleavage of human embryos for 5 days *in vitro* following intracytoplasmic sperm injection (ICSI) and maturation in no hormones, rFHS (1 IU/ml) alone or rFSH (1 IU/ml) in combination with rLH (10 IU/ml). Results are expressed as mean ± SEM.

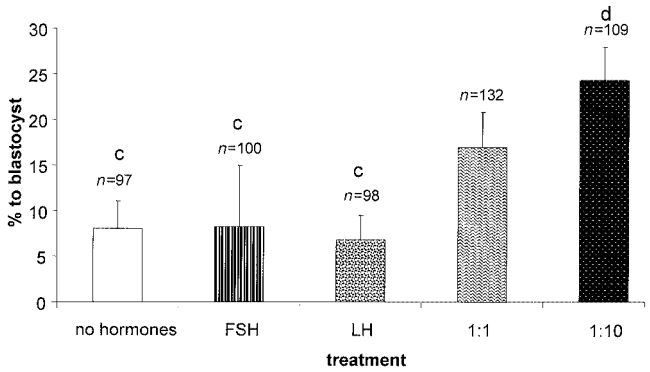

Figure 3. Development of bovine embryos to blastocyst on day 7 of culture following maturation in no hormones, rFSH (1 IU/ml) alone, rLH (1 IU/ml) alone, rFSH (1 IU/ml) in combination with rLH (1 IU/ml) or rFSH (1 IU/ml) in combination with rLH (10 IU/ml). Results are expressed as mean ± SEM and *n* represents the total number of oocytes from several replicates. Bars labelled c and d are significantly different at $P < 0.05$.

embryos were transferred to five patients. However, no pregnancies resulted.

The concomitant addition of either a 1:1 or 1:10 ratio of rFSH and rLH improved the development of bovine embryos to blastocyst stage (Figure 3). Furthermore, the improvement in bovine embryonic development was significantly ($P < 0.05$) greater in the 1:10 culture group compared to the no hormone and FSH alone and LH alone groups (Figure 3).

The in-vitro development of mouse embryos to blastocyst stage was increased when mouse oocytes were exposed to a 1:1 or a 1:10 ratio of rFSH and rLH (Table I). However, this increase in mouse blastocyst growth in the 1:1 and 1:10 groups was not significantly different ($P = 0.767$) from either the no hormone or FSH alone and LH alone groups (Table I).

Differential labelling of cells

The inner cell mass of a blastocyst gives rise to the embryo-proper and is known to be affected by a variety of treatments (Sherman, 1979). Furthermore, total cell number is representative of cleavage rate and developmental competence. The treatment with a 1:10 ratio of rFSH and rLH was shown to

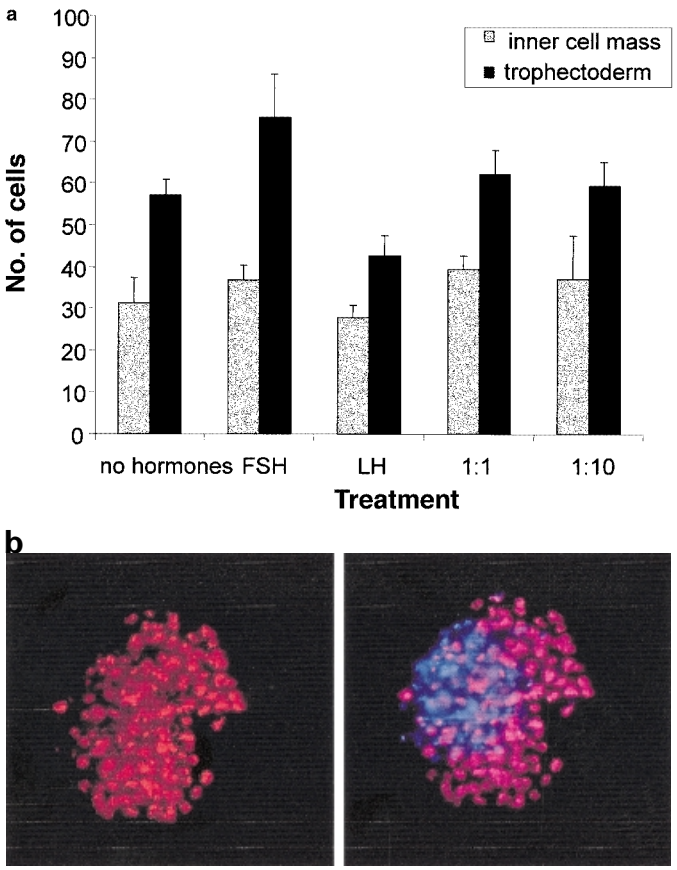

Figure 4. (**a**) Number of trophectoderm and inner cell mass cells in bovine blastocyst on day 7 following maturation in no hormones, rFSH (1 IU/ml) alone, rLH (1 IU/ml) alone, rFSH (1 IU/ml) in combination with rLH (1 IU/ml) or rFSH (1 IU/ml) in combination with rLH (10 IU/ml). Results are expressed as mean ± SEM. (**b**) A bovine blastocyst differentially stained with propidium iodide and bisbenzimide. Trophectoderm cells appear red/pink under a blue/green light using a 350–460 nm filter (left panel) and inner cell mass cells appear blue using a 340–380 nm wavelength filter (right panel). All embryos were viewed under a UV light on a Leitz fluorescent microscope at ×200 original magnification.

increase the number of bovine embryos developing to the blastocyst stage and differential staining was employed to investigate whether gonadotrophin treatment with a 1:10 ratio of rFSH:rLH also affected the number of cells in the resultant blastocysts.

There was no significant difference in the number of bovine TE cells ($P = 0.243$) and the number of ICM cells ($P = 0.137$) in any of the treatment groups investigated (Figure 4a). ICM cells were counted under UV light using a 340–380 nm wavelength filter and appeared blue in colour. The TE cells were counted under a blue/green light using a 350–460 nm filter and appeared red/pink in colour (Figure 4b).

Human and murine embryos were not assessed by differential staining as human embryos, cleaving to the blastocyst stage, were transferred to the patients and murine embryos treated with the different gonadotrophins did not display any notable differences in embryonic development.

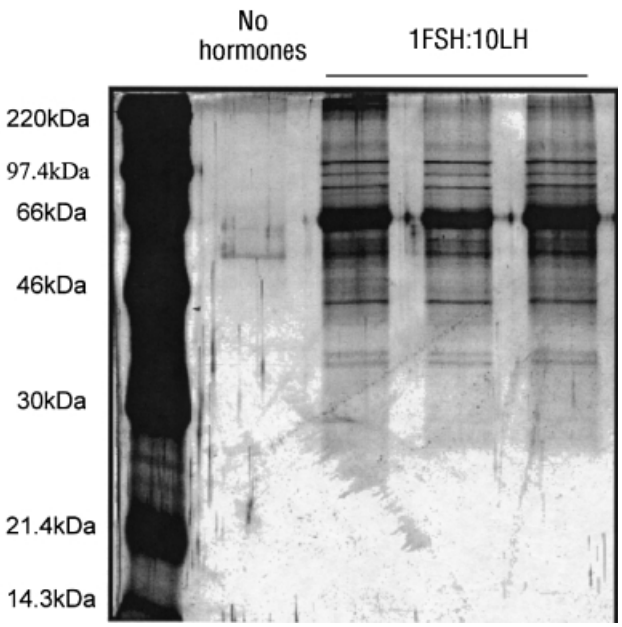

Figure 5. Cytoplasmic protein profile of single metaphase II in-vitro matured human oocytes: one-dimensional 12% SDS-polyacrylamide gel of oocyte proteins stained with silver nitrate. Each lane contains a single denuded human oocyte.

Investigation of oocyte proteins

Following the lysis, solublization and electrophoresis of the entire oocyte, the resulting protein profile includes cytoplasmic, membrane and nuclear proteins. However, it is important to note that staining with silver nitrate only allows for the visualization of the most abundant proteins.

Due to the limited numbers of human oocytes donated for research, comparisons of oocyte proteins in human oocytes were made between the no hormone group and 1:10 group as these groups displayed the greatest variances in maturation, and developmental competence. SDS–PAGE was repeated three times using human oocytes and resulted in a consistently reproducible finding. In comparison to oocytes matured without gonadotrophin supplementation, oocytes matured in a 1:10 ratio of FSH to LH demonstrated a dramatic increase in protein content (Figure 5).

SDS–PAGE of bovine and murine oocyte proteins was repeated 5 times and the protein content of bovine and murine oocytes at the metaphase II stage was consistently similar in the no hormone, FSH alone, LH alone, 1:1 and 1:10 groups (Figures 6 and 7).

All oocytes were denuded of cumulus cells prior to solubilization in Laemmli sample (Laemmli, 1970) buffer for SDS–PAGE. However, a maturation medium control and human, bovine and murine cumulus cell controls were run on 12% polyacrylamide gels to identify whether any adherent cumulus cells or maturation medium contamination could have contributed to the protein profiles observed.

Ten thousand human, 20 000 murine and 30 000 bovine cumulus cells are required to generate a profile of proteins ranging in molecular weight from 220 to 21.5 kDa. A protein profile cannot be generated with <1000 human, <200 murine and <3000 bovine cumulus cells (data not shown), indicating

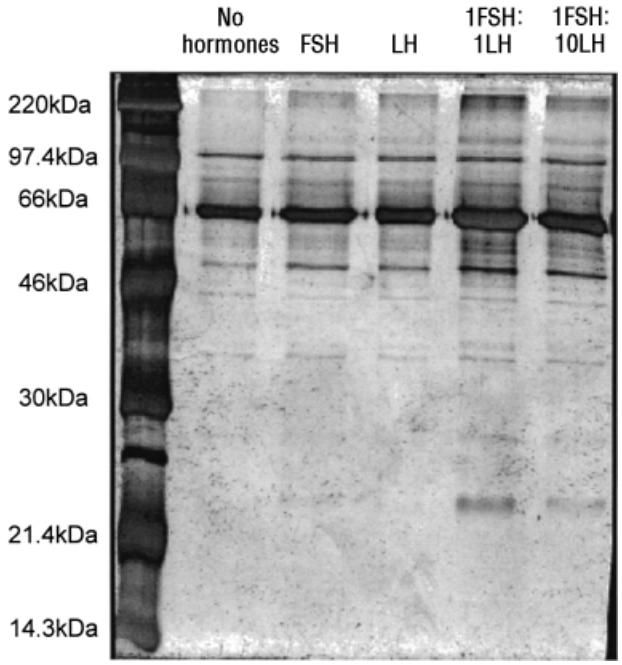

Figure 6. Cytoplasmic protein profile of groups of three metaphase II in-vitro matured bovine oocytes: one-dimensional 12% SDS-polyacrylamide gel of oocyte proteins. Each lane contains three denuded bovine oocytes.

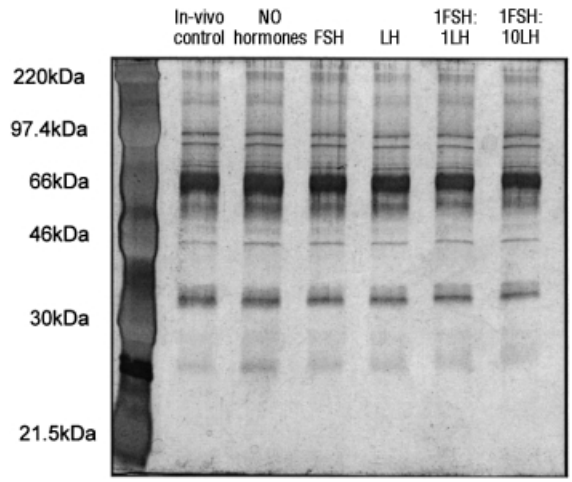

Figure 7. Cytoplasmic protein profile of groups of 10 metaphase II murine oocytes: one-dimensional 12% SDS-polyacrylamide gel of oocyte proteins stained with silver nitrate. Each lane contains 10 denuded murine oocytes.

that one or two adherent cumulus cells could not have contributed to the oocyte protein profiles observed.

The maturation medium control revealed a major band in the region of 60 kDa (data not shown). This band was also seen in the lanes containing cumulus cells and most probably represents albumin contamination, as albumin is present in all the handling medium to which oocytes are exposed.

Discussion

Mammalian oocytes spontaneously mature *in vitro*, following liberation from the follicle (Pincus and Enzmann, 1935). However, the kinetics of maturation displays species variations. Approximately 40% of murine and bovine oocytes will begin meiotic resumption within 1–2 h of release from the follicle (C.Anderiesz, unpublished data). Human oocytes, by comparison, can remain at the GV stage for up to 24 h (Trounson *et al.*, 1994). Since the LH surge precedes meiotic resumption the addition of rLH to culture medium in this study was timed to precede the spontaneous resumption of maturation in the individual species. Consequently, bovine and murine oocytes received rLH supplementation at the onset of culture (0 h) and human oocytes received their rLH addition after 24 h of culture. In addition, the concentrations of gonadotrophins used in this study, although not physiological, have been previously used for oocyte in-vitro maturation (Chanson *et al.*, 1997; C.Anderiesz, unpublished data) and elevated concentrations of LH have been demonstrated to enhance the viability of in-vitro matured oocytes (Brackett *et al.*, 1989). Furthermore, up to 100 IU/ml of LH can be used during in-vitro maturation without compromising embryonic development (Keefer *et al.*, 1993).

It is well established that primary human oocytes are capable of undergoing meiotic maturation *in vitro* (Edwards, 1965; Cha *et al.*, 1991; Trounson *et al.*, 1994; Barnes *et al.*, 1995, 1996; Russell *et al.*, 1997). However, subsequent embryonic development of in-vitro matured human oocytes is compromised as oocytes demonstrate retardation and cessation of cleavage divisions (Trounson *et al.*, 1996, 1998) and this may be related to the poor pregnancy outcomes observed.

In this study, the improvements in human oocyte maturation in the presence of FSH were similar to those reported previously (Durinzi *et al.*, 1997).

It has been demonstrated (Armstrong *et al.*, 1991) that human oocytes undergo normal cleavage following the addition of gonadotrophins to culture medium and human embryonic development was improved in this study by the maturation of oocytes with rFSH (1 IU/ml) and a 10 times concentration of rLH (10 IU/ml) as only human oocytes matured in rFSH and high concentrations of rLH demonstrated normal embryonic cleavage beyond day 2 of culture. The inclusion of a 1:10 ratio of FSH:human chorionic gonadotrophin (HCG) has been used for the routine culture of immature human oocytes (Wynn *et al.*, 1998). However, to date, no comparisons have been made in regard to the developmental potential of human oocytes treated with a 10 times concentration of LH. Importantly and interestingly, this study demonstrates an improvement in the embryonic development of in-vitro matured human oocytes and extends earlier work (Zhang *et al.*, 1993) in which it was demonstrated that human embryonic cleavage during the first 24–32 h could be improved by the addition of human menopausal gonadotrophin (HMG) to culture medium. However, small sample numbers have placed constraints on deriving statistical significance.

In this study, the similarity in maturation and embryonic development of bovine oocytes matured in either rFSH or rLH alone or without gonadotrophins is consistent with previous findings (Keefer *et al.*, 1993). In contrast, earlier studies (Zuelke and Brackett, 1990) demonstrated that LH alone was of benefit to the fertilization and embryonic development of bovine oocytes. This discrepancy in results may be attributable

to the LH preparation used. In the aforementioned study, the LH preparation was contaminated with FSH and thyroid stimulating hormone (TSH) and these and other contaminants may have influenced the results (Zuelke and Brackett, 1990).

The enhancement in maturation and developmental ability of oocytes exposed to both FSH and LH *in vitro* is in keeping with previously published results in both porcine (Singh *et al.*, 1993) and bovine (Saeki *et al.*, 1991) oocytes. However, single or combined gonadotrophin treatments had no effect on actual bovine embryonic cell number. It has been previously reported that bovine embryos cultured *in vitro* have a lower embryonic cell number than embryos that have developed *in vivo* (Iwasaki *et al.*, 1990; de la Fuente and King, 1997). In comparison to the bovine blastocyst grown *in vivo* (Iwasaki *et al.*, 1990; de la Fuente and King, 1997) the in-vitro cultured blastocysts in this study have a reduced total cell number.

The number of ICM cells observed in the present study was similar to those previously reported for in-vitro cultured bovine embryos (Iwasaki *et al.*, 1990; de la Fuente and King, 1997). However, the ratio of ICM cells to total cell number seen in the *in vitro* cultured bovine blastocysts (Iwasaki *et al.*, 1990; de la Fuente and King, 1997) was lower than observed in the current study. The high ratio of ICM cells to total cell number observed appears to be due to a low TE cell number which in turn may be attributable to lysis of TE cells during permeabilization with the antibody-mediated compliment.

Interestingly, bovine embryonic development to the blastocyst stage was significantly higher when oocytes matured with rFSH together with a high concentration of rLH than either rFSH or rLH alone or no hormones. The beneficial effect of a high concentration of LH on bovine embryonic development, to the 8-cell stage, has been reported previously (Brackett *et al.*, 1989). FSH and LH receptors are present on cumulus cells (Lawrence *et al.*, 1980; Channing *et al.*, 1981; Shima *et al.*, 1987; Bao *et al.*, 1997) and thus, FSH and LH elicit their effects via cumulus mediated interactions. However, the exact mechanisms by which gonadotrophins influence embryonic development are unknown. The present studies suggest that gonadotrophins may improve oocyte viability by influencing the oocyte's protein content. FSH and LH have previously been shown to influence the protein synthetic capacity of oocytes (Moor *et al.*, 1985). Human oocytes treated with a 1:10 ratio of rFSH:rLH demonstrated a greater amount of proteins than oocytes matured without gonadotrophins. Since present data together with previous findings (Moor *et al.*, 1985) suggest that FSH and LH are capable of stimulating protein synthesis in oocytes, the absence of gonadotrophins in culture serves to explain the reduced protein content observed in human oocytes matured in the 'no hormone' group.

Human oocytes matured in the absence of gonadotrophins display a very low protein content and the increase in human oocyte proteins, in the presence of gonadotrophins, may therefore be both quantitative and qualitative. However, silver nitrate staining only permits visualization of the most abundant proteins; thus, the gonadotrophin treatment may have also induced qualitative changes in proteins, but these may have been below the detection limit of silver nitrate staining. Additionally, LH has been shown to increase the activity of the TCA cycle and hence regulate the nutritional environment of the oocyte (Zuelke and Brackett, 1992, 1993). Therefore, LH may be able to improve embryonic development by modulating the oocyte's nutritional environment. However, this was not directly investigated and would require verification.

The differences in protein profiles were not observed in gonadotrophin-treated murine or bovine oocytes. Murine oocytes were exposed to PMSG *in vivo*. PMSG administration has been demonstrated to induce changes in protein synthesis (Moor *et al.*, 1985). In particular PMSG administration results in protein translationary changes that are normally associated with maturation (Moor *et al.*, 1985). In this study, pre-treatment with PMSG may have negated the synthetic effect of rFSH and rLH *in vitro*, by eliciting translationary changes in the murine oocytes *in vivo*.

Bovine oocytes were retrieved from follicles >2 mm. Bovine oocytes retrieved from follicles of this size have been shown to be developmentally competent (Pavlok *et al.*, 1992), suggesting that the normal sequence of events leading to maturation and early embryonic development can be activated *in vitro*. Thus, a similar pattern of proteins would be expected to be seen in both the no hormone and gonadotrophin treatment groups and any differences in protein profiles would be subtle. However, small changes in the protein profiles may not have been detected due to the pooling of oocytes for analysis and the inability of silver nitrate staining to detect small translationary alterations. Previous studies in the mouse have revealed that the inclusion of FSH in maturation medium improves fertilization, fetal development and increases the frequency of preimplantation development (reviewed in Armstrong *et al.*, 1991; Merriman *et al.*, 1998). However, consistently similar outcomes were obtained for the maturation, fertilization and embryonic development of murine oocytes matured without hormones and those matured with either the individual or combined additions of rFSH and rLH. The lack of response to in-vitro gonadotrophin supplementation may be related to the pre-treatment of mice with PMSG. PMSG possesses both FSH and LH activity and prior exposure of COCs to these exogenous gonadotrophins may have served to limit or negate entirely the effects of rFSH and rLH *in vitro*. Indeed, priming with gonadotrophins has previously been demonstrated to improve meiotic maturation, fertilization and embryonic development of rodent oocytes and evidence suggests that it may be due to the stimulation of cytoplasmic maturation (reviewed in Armstrong *et al.*, 1991).

Murine oocytes in this study were treated with a five times lower concentration of rFSH and rLH than the bovine and human oocytes. It is unlikely that this lower concentration of gonadotrophins could have contributed to the poor developmental response of murine oocytes as it has been previously demonstrated that rodents can respond to between 50 and 1000 times less gonadotrophins than bovine oocytes (Brackett *et al.*, 1989; Saeki *et al.*, 1991; Törnell *et al.*, 1995).

The present study demonstrates that the joint supplementation of rFSH (1 IU/ml) and a high concentration of rLH (10 IU/ml) to in-vitro maturation medium improves the embryonic development of human and bovine oocytes. Furthermore, the improvement in developmental competence

observed in the presence of both rFSH and rLH, underscores the important action of both gonadotrophins in the regulation of oocyte maturation and embryonic development *in vitro* and provides a sound rationale for the inclusion of these gonadotrophins in in-vitro maturation medium.

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