ANIMAL EXPERIENCE

Development of a Well-Defined Medium for the In Vitro Maturation of Immature Bovine Cumulus–Oocyte Complexes

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Purpose: Our purpose was to develop a well-defined medium for the in vitro maturation (IVM) of immature bovine cumulus–oocyte complexes (COC).

Methods: The COC were cultured in the presence of three protein supplementations: fetal bovine serum (FBS), bovine serum albumin, and Synthetic Serum Substitute. The embryos obtained after in vitro fertilization of IVM oocytes were cocultured with Vero cells and their development to the morula and blastocyst stages was studied.

Results: When FBS was absent from the IVM medium, a significantly lower fertilization rate was observed, followed by a decrease in the percentage of embryos reaching the blastocyst stage. When FBS was replaced by a defined protein supplementation, the best results were obtained with Synthetic Serum Substitute.

Conclusions: Adequate protein supplementation of the IVM medium optimizes the fertilization rate and the development of bovine IVM oocytes. The implication of these results in the human field is discussed.

KEY WORDS: In vitro maturation; oocyte; follicle; ovary; bovine.

INTRODUCTION

In clinically difficult cases, such as poor responders and PCO patients, whose reactions to ovarian stimulation are either too weak or excessive, an alternative approach to superovulation, consisting of immature oocyte collection and in vitro maturation (IVM), has been developed by Trounson *et al.* (1). Instead of priming the woman with follicle stimulating hormone (FSH) and human chorionic gonadotropin (hCG) to induce follicular maturation and ovulation, immature oocytes were recovered during the follicular phase and matured in vitro in a specially designed culture medium containing fetal calf serum (FCS), human menopausal gonadotropins (hMG), hCG, and estradiol (E₂). Mature oocytes were inseminated with the husband's sperm and the best embryos transferred to the patient. In 1993, the first baby using this technique was born. Several other pregnancies were achieved after modification of the technique including intracytoplasmic sperm injection (ICSI), assisted hatching, and in vitro culture of the embryos to the blastocyst stage (2,3). Unfortunately, the pregnancy potential of the in vitro matured oocytes is still low, as, with a few exceptions (4,5), only 2–10% of treated patients achieve a pregnancy (6–9). Embryos deriving from in vitro matured oocytes showed cleavage retardation and blockage around the eight-cell stage at a higher frequency than those obtained after superovulation. Both incomplete maturation of cell cycle regulators and inadequate culture conditions have

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been associated with these observations (6). Therefore, the establishment of in vitro maturation protocols that would promote and maintain competence of the cumulus–oocyte complexes (COC) for fertilization and embryogenesis is still required (10). In this respect, the development of a well-defined maturation medium, in particular, the use of an efficient and safe protein supplementation, would greatly improve IVM techniques. There has been a trend in recent years to replace poorly known serum preparations by more defined proteins such as bovine serum albumin (BSA), human serum albumin (HSA), synthetic serum preparations, or plasma protein fractions (11). Several considerations have motivated these changes, but the two main concerns are potential viral or prion contamination and variability of the serum preparations. Clearly, with the recent appearance of bovine spongiform encephalopathy, the use of bovine-derived proteins for human IVF has to be avoided in human IVF procedures and be replaced by a HSA of controlled origin.

Large inert molecules, such as polyvinyl alcohol (PVA), have also been used to exclude any protein from culture media (12–16). To our knowledge, these completely defined media have never been tested for the IVM of human oocytes. However, recent data of Holm *et al.* (16) showed that completely defined IVM media reduced the cleavage and blastocyst rates of bovine COC. The presence of serum or BSA during the IVM period was essential for normal fertilization and subsequent high blastocyst development.

As the use of human material for research purposes is questionable from an ethical and practical standpoint, the bovine system was selected as a model for the development of the IVM techniques in our unit. Indeed, IVM was developed initially as an alternative to superovulation for embryo production in cattle (17) and most of the pioneer experiments were performed using this bovine system (1).

The aim of this study was to set the conditions for successful IVM and IVF of bovine antral immature oocytes, allowing optimal rates of fertilization and blastocyst development, using a well-defined IVM medium based on the protein supplementation commonly used in the field of human IVF.

MATERIALS AND METHODS

Recovery of the COC

The cow ovaries were obtained from the local slaughterhouse. Immediately after collection, they were placed in sterile phosphate-buffered saline (PBS; supplemented with 50 μ g/ml gentamicin) and maintained at 39°C in a water bath. The ovaries were transported in an isotherm box ($\sim$ 35–39°C) to the laboratory within 2–3 hr and washed in sterile PBS. The follicular fluids were aspirated gently with a needle (19 G) and a syringe (5 ml) from small antral follicles (2 to 8 mm) and placed in 16-ml sterile conical tubes (Nunc, Gibco BRL, Paisley, Scotland) maintained at 39°C. The sedimented COC were transferred blind after 30 min into 2 ml of HEPES-buffered medium 199 (M199-HEPES; Gibco BRL), supplemented with 10% heat-inactivated fetal calf serum (FBS; Gibco BRL), 50 μ g/ml gentamicin and adjusted to a final osmolarity of 290 ± 2 mOsm. COC were recovered under a stereomicroscope and rinsed several times with M199-HEPES.

In Vitro Maturation (IVM) of the COC

The COC were pooled in two groups, group A (classes 1–3) and group B (classes 4–6), following the morphological criteria of Blondin and Sirard (18) (Table I). Preliminary experiments showed that group B oocytes were hardly ever fertilized after IVM and were thus discarded. Three IVM media

Table I. Classification of Bovine Oocyte–Cumulus Complexes [from Blondin and Sirard (18)]

Class	Group	No. of cumulus layers	Expansion of cumulus	Texture of ooplasm
1	A	≥ 5	Compact	Homogenous
2	A	≥ 5	Compact	Dark zone around periphery
3	A	≥ 5	Slight expansion in outer layers	Slight granulations
4	B	≥ 5	Full expansion with dark clumps, atretic	Heavy granulations
5	B	1	Only corona radiata	Variable
6	B	0	No cumulus	Variable

were tested. (a) Bicarbonated M199 medium (M199-HCO₃; Gibco BRL), supplemented with 10% heat-inactivated FBS, 50 μ g/ml gentamicin and adjusted to a final osmolarity of 290 ± 2 mOsm, was used as a control. FBS was replaced by either (b) 1% BSA (Sigma A3311) with 0.2% Synthetic Serum Replacement medium (SSR2; Medi-Cult, Copenhagen, Denmark) or (c) 10% Synthetic Serum Substitute (SSS; Irvine Scientific, Santa Ana, CA). Recombinant FSH (Gonal-F; Serono, Geneva, Switzerland; 1 IU/ml), recombinant LH (LHADI; Serono; 1 IU/ml), and estradiol (E₂; Sigma E2758; 1 μ g/ml) were added to all IVM media. The COC were placed in 0.5 ml of the tested IVM medium in four-well culture dishes (Nunc; Gibco BRL). IVM was carried out by culturing the COC under standard incubation conditions (39°C, 5% CO₂; 95% air) in a humidified incubator (BB 16; Heraeus, Hanau, Germany) for 22–24 hr.

Preparation of the Bull Sperm

Straws of frozen bull sperm were provided by the Swiss Federation of Artificial Insemination (Mülligen, Switzerland). After preliminary experiments, the sperm of one bull was selected, for its ability to lead to high segmentation rates. Just prior to the end of the IVM period, the spermatozoa were prepared for fertilization. One straw (0.5 ml) was rapidly thawed at 37°C. A 5- μ l aliquot was deposited in a counting chamber (Conception Technologies, San Diego, CA); the concentration and motility were determined with a computer-assisted sperm analyzer (HTM; Hamilton Thorne Research, Beverly, MA). The thawed sperm suspension was layered on top of a Percoll gradient (1 ml 90% and 1 ml 50% in isoosmotic HAM-F-10 medium), which was then centrifuged for 10 min at 300g. The pellet of motile spermatozoa was recovered in 500 μ l of SPZ-TALP medium (19) containing 10 μ g/ml heparin. After reassessment of motile spermatozoa concentration, the sperm suspension was incubated under standard conditions for 1 hr.

In Vitro Fertilization (IVF) of the Mature COC

In vitro matured oocytes were transferred in 0.9 ml fresh TALP-FIV medium, supplemented with heparin (10 μ g/ml), hypotaurine (10 μ M), and epinephrine (1 μ M) (19). An aliquot of the washed spermatozoa suspension was then added to each well,

containing 20–40 COC, to achieve a final concentration of about 1 million motile spermatozoa per ml. Gametes were then incubated under standard conditions for 17–19 hr. The rate of fertilization was calculated as the percentage of inseminated COC reaching the two-cell embryo stage.

In Vitro Culture of the Embryos

After IVF, the embryos were denuded by vortexing (2 min) and rinsed several times in Menezo B2 medium (BioMérieux SA, Marcy-l'Etoile, France) supplemented with 10% FBS. The embryos were co-cultured (7 to 8 days) in the presence of Vero cells (20,21) and their development through the morula and blastocyst stages was monitored. The blastocyst formation rate was calculated as the percentage of two-cell embryos reaching the blastocyst stage.

Preliminary Experiments

Over 1 year, 46 experiments were carried out to select the most appropriate conditions for COC recovery, IVM, IVF, and embryo culture up to the blastocyst stage. Five hundred thirty-eight ovaries yielding 2865 COC (an average of 5.3 COC per ovary) were used. Several experiments were necessary to test different COC recovery protocols: various needle sizes (25 and 19 gauge), syringe volumes (2, 5, and 10 ml), and antibiotics (penicillin, streptomycin, gentamicin). The relationship between COC morphology and follicle diameter was studied and the 2- to 8-mm follicles were shown to be the best source for immature COC. Different IVM periods (18, 24, 36, and 48 hr) were tested and the optimum was found to be around 24 hr. Various fluorescent DNA staining protocols [DAPI (22), Hoechst 33342 (23)] were tested and the Hoechst 33342 was retained for its greater fluorescing stability. Several sperm capacitation techniques (swim-up separation, differential centrifugation, Percoll gradients, heparin at different concentrations, calcimycin, etc.) and capacitation media were tested. The best results were obtained using a Percoll gradient purification technique and capacitation in the presence of heparin in SPZ-TALP (19). Furthermore, two coculture methods were tested to improve the development of the embryos up to the blastocyst stage by using, as feeder cells, either bovine granulosa cells prepared weekly or Vero cells. The best results were achieved with the Vero cell coculture method.

Statistical Analysis

As only a limited number of ovaries (about 10 to 20) were obtainable at the same time at the slaughterhouse, a mean number of 62 ± 14 COC ($N = 22$; range, 43–96) was available for each assay. To take into account the variation of the fertilization rates between batches of ovaries, a control experiment was always carried out with half of the COC. For each IVM medium tested, at least 150 COC were cultured. This number was chosen by taking into account (a) the number of COC available for each experiment (about 30 for the control and tested conditions, respectively) and (b) the number of replicates ($N \geq 6$) necessary to reach this number over a reasonable period of time (one experiment per week during a period of 6 months). The data from the different duplicates were pooled and statistically analyzed using 2×2 contingency tables and the χ^2 test (StatView for Macintosh). Differences of $P < 0.05$ were taken to be significant.

RESULTS

Biological Variability of the Ovaries

The quality of the ovaries varied from one experiment to the other, depending on the period of the year, the age of the cows, and the mixture of breeds. For example, the mean results of five experiments carried out at the end of the development phase are presented in Table II. Whereas the fertilization rates, based on the number of in vitro matured oocytes reaching the two-cell stage after insemination, varied from 46.5 to 93.3%, less variation was observed for the rates of development to the blastocyst stage (21.4 to 30.0%).

Protein Supplementation of the IVM Media

Protein supplementation of the IVM media is usually achieved by adding FBS (2). Three series of ex-

periments were carried out to compare the cleavage and blastocyst formation rates after maturation of the COC (a) in the absence of FBS, (b) in the presence of Synthetic Serum Replacement medium (SSR2) and BSA, and (c) in the presence of Synthetic Serum Substitute (SSS). Half of the oocytes were matured in the presence of FBS as a control to rule out (or eliminate) uncontrollable variations of fertilization rates from one experiment to another. All IVM media were supplemented with a combination of LH, FSH, and E_2 . In the absence of FBS (Fig. 1), the fertilization rate was decreased significantly ($P = 0.0137$), from 45.2 to 35.9%, but the blastocyst formation rate was only slightly affected (19.7 versus 14.9%, NS). When the FBS was replaced by BSA and SSR2 (Fig. 2), no differences were observed between the two groups (fertilization rates, 35.4 versus 31.0%, NS; blastocyst formation rates, 22.8 versus 22.6%, NS). In the presence of SSS (Fig. 3), the fertilization rate was similar to that of the control (31.5 versus 33.1%), but the percentage of the two-cell-stage oocytes reaching the blastocyst stage increased from 23.4 to 33.3% ($P = 0.261$, NS).

DISCUSSION

The maturation of immature COC is probably the most critical in vitro step affecting subsequent embryo development to the blastocyst stage (24). The medium used for oocyte maturation can strongly affect the developmental capacity of in vitro produced bovine embryos, as shown by Bavister *et al.* (25,26), who compared seven culture media. Serum preparations and BSA are among the most common protein supplements of IVM media (25,27–29). The postulated role of serum proteins in embryo culture appears to be that of a fixed nitrogen source, a chelator of toxic metal ions, and an antioxidant (30). From a more practical point of view, if there is no protein in the culture

Table II. Biological Variability of the Ovaries: Cleavage Rate and In Vitro Culture of Bovine Oocyte–Cumulus Complexes to the Blastocyst Stage During Five Consecutive Experiments

Experiment No.	<i>N</i>	No. of 2-cell stage (fertilization rate; %)	No. of blastocyst stage from 2-cell stage (%)
1	30	28 (93.3)	6 (21.4)
2	31	26 (83.9)	6 (23.0)
3	20	18 (90.0)	4 (22.2)
4	25	21 (84.0)	5 (23.8)
5	43	20 (46.5)*	6 (30.0)
Total (mean $\pm$ SD)	149	113 (79.5 $\pm$ 18.9)	27 (24.1 $\pm$ 3.4)

* Experiment 5 significantly different from each of the other experiments ($P = 0.0001$).

Fig. 1. Effect of the absence of FBS in the maturation medium on the fertilization and blastocyst formation rates.

Fig. 2. Effect of BSA and SSR2 in the maturation medium on the fertilization and blastocyst formation rates.

Fig. 3. Effect of SSS in the maturation medium on the fertilization and blastocyst formation rates.

medium, the COC and oocytes are difficult to handle, sticking to plastic and glassware (30). However, serum contains hormones, growth factors, vitamins, chelators of heavy metal ions, peptides, proteins, and an array of defined and nondefined molecules (31), whose biological activity may differ considerably from one lot to another (32,33). Serum is thus not a good candidate for the formulation of a well-defined culture medium. Better-controlled culture systems are necessary prerequisites for the study of molecular mechanisms involved in oocyte maturation, but also fertilization or embryo development. Animal serum is also a potential source of infections, especially of viral or prion origin. For these reasons, the presence of serum, though useful when setting up initial cell culture procedures, can ultimately cause insurmountable complications and ambiguities. Recently, several protein supplementation media have been commercialized. For example, Medicult's chemically defined SSR2 used with highly purified HSA (10 g/L) is an extremely effective protein supplement for human IVF work (11,34). Another such defined medium is synthetic human tubal fluid (HTF) supplemented with HSA (35). More recently SSS was introduced as a protein supplement for human IVF

procedures. SSS is composed of 84% HSA, 16% α - and β -globulins, with no more than 1% γ -globulins (30,36).

The results of the present study confirm previous observations showing that protein supplementation is beneficial for the IVM of bovine follicular COC (28,37–40). Indeed, oocytes matured in the absence of FBS presented lower fertilization rates and slightly poorer development to the blastocyst stage (Fig. 1) as reported by other authors (31,41). Nevertheless, maturation in the presence of hormones (LH, FSH, and E_2) and without FBS allowed a substantial number of IVM oocytes to be fertilized normally and to develop to the blastocyst stage as reported previously (39,42,43). The requirement for hormones, however, remains controversial, as similar fertilization and blastocyst formation rates were obtained in their absence (fertilization, 46.5 versus 48.2%, NS; blastocyst formation, 21.0 versus 23.3%, NS; data not shown). These data clearly show that hormones were not inhibitory at the concentration used (1 IU/ml FSH, 1 IU/ml LH, and 1 μ g/ml E_2). When the FBS was replaced by BSA and SSR2, identical results were observed, in terms of fertilization and blastocyst formation rates. Since the biological

activity of commercially available BSA may differ remarkably from one lot to another, SSS, a protein supplement specifically developed for human IVF procedures, was tested as an alternative. With this protein supplementation, results were improved, especially in terms of blastocyst formation rate. SSS has already been used for the IVM of immature human oocytes (3), for the IVF of metaphase II human oocytes (11,30,44,45), and for the culture of human embryos (11,36,46–48). However, no comparative studies have been published so far on IVM. SSS has also been found to be superior to HSA alone when murine embryos were cultured up to the hatched blastocyst stage (49). Further comparative experiments will be necessary in the field of human IVM to confirm the encouraging results obtained with SSS on the bovine model system. Finally, Svalander *et al.* (50) showed very recently that recombinant human albumin (rHA) supports in vitro fertilization, embryo development, and blastocyst hatching in the mouse. rHA is structurally identical to HSA and free from viral or prion contamination, has greater homogeneity, and has a high batch-to-batch consistency. The use of recombinant protein to develop completely defined culture media seems to be very promising.

CONCLUSION

We confirm that protein complementation of the IVM medium is necessary to optimize the fertilization rate and development to the blastocyst stage of immature COC obtained from ovaries of slaughtered cows. In the presence of E₂ and recombinant gonadotropins (FSH and LH), the serum (FBS) can be replaced by a mixture of defined proteins without affecting the fertilization rate and the development of bovine IVM oocytes. SSS yields better results than FBS or SSR2 plus BSA. Now, further experiments with this chemically defined medium should be carried out in the field of human IVM. The use of a bovine model system in a human IVF laboratory is very convenient for studying different aspects of mammalian oocyte maturation. The bovine system was found to be an excellent model from which to learn the basic IVF techniques, and it could become a teaching tool for biologists interested in human IVF. It is actually too early to tell whether IVM techniques will revolutionize the treatment of infertility (51). In any case, major increases in the yield and viability of human IVM/IVF

embryos will require a better understanding of the optimal conditions for supporting oocyte maturation, and more research is needed in this new and promising field.

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