Western University
Scholarship@Western

Obstetrics & Gynaecology Publications | Obstetrics & Gynaecology Department

4-1-1994

Preimplantation development of in vitro-matured and in vitro-fertilized ovine zygotes: comparison between coculture on oviduct epithelial cell monolayers and culture under low oxygen atmosphere.

A J Watson

P H Watson

D Warnes

S K Walker

D T Armstrong

See next page for additional authors

Follow this and additional works at: https://ir.lib.uwo.ca/obsgynpub

Part of the Obstetrics and Gynecology Commons

Citation of this paper:
Watson, A J; Watson, P H; Warnes, D; Walker, S K; Armstrong, D T; and Seamark, R F, "Preimplantation development of in vitro-matured and in vitro-fertilized ovine zygotes: comparison between coculture on oviduct epithelial cell monolayers and culture under low oxygen atmosphere." (1994). *Obstetrics & Gynaecology Publications*. 85.
https://ir.lib.uwo.ca/obsgynpub/85

Authors
A J Watson, P H Watson, D Warnes, S K Walker, D T Armstrong, and R F Seamark

This article is available at Scholarship@Western: https://ir.lib.uwo.ca/obsgynpub/85

BIOLOGY OF REPRODUCTION 50, 715–724 (1994)

Preimplantation Development of In Vitro-Matured and In Vitro-Fertilized Ovine Zygotes: Comparison between Coculture on Oviduct Epithelial Cell Monolayers and Culture under Low Oxygen Atmosphere[1]

ANDREW J. WATSON,[2,3] PATRICIA H. WATSON,[4] DEIRDRE WARNES,[5] SIMON K. WALKER,[6]
DAVID T. ARMSTRONG,[3,5] and ROBERT F. SEAMARK[5]

Department of Obstetrics and Gynaecology, University of Western Ontario[3]
London, Ontario, Canada N6A 5A5
Lawson Research Institute,[4] St. Joseph's Health Centre, London, Ontario, Canada N6A 4V2
Department of Obstetrics and Gynaecology,[5] University of Adelaide, Adelaide, South Australia 5000
South Australia Research and Development Institute,[6] Turretfield Research Center, South Australia 5350

ABSTRACT

The roles of medium composition, serum source, embryo coculture, and culture under low O_2 conditions on the development of in vitro-matured and in vitro-fertilized (IVMF) ovine zygotes were investigated in three separate experiments. In the first experiment, the proportion of cocultured IVMF zygotes developing to the blastocyst stage was significantly higher (38.0% vs. 3.5%; $p < 0.05$) than that of non-cocultured zygotes treated within three embryo culture media (TCM-199 + 10% fetal bovine serum [FBS]; bicarbonate-buffered, glucose-free synthetic oviduct fluid medium [mod-SOFM] + 10% FBS; and bicarbonate-buffered BSA-free Tyrode's salt solution [mod-TALP] + 10% FBS) under a 5% CO_2 atmosphere in air. In a second experiment, a significantly higher ($p < 0.05$) proportion of cocultured zygotes placed in TCM-199 medium survived to the blastocyst stage (37.4% blastocysts vs. 23.4% in mod-SOFM). No significant effect of serum (FBS vs. human serum [HS]) was observed on embryonic development, but coculture was confirmed to exert a significant influence on development to the blastocyst stage. In the final experiment, survival of the embryo under a reduced oxygen (5% CO_2:5% O_2:90% N_2) atmosphere was investigated. In contrast to results in the initial experiments, embryonic survival was significantly higher ($p < 0.05$) in the non-cocultured treatment groups (21.9% blastocysts vs. 0.4% for cocultured zygotes). Serum source also had a significant ($p < 0.05$) influence upon the development of non-cocultured zygotes: 32.3% of zygotes cultured with HS progressed to the blastocyst stage vs. 11.5% of zygotes cultured in FBS-supplemented medium. These results have characterized two distinct culture environments, each capable of supporting the development of high frequencies of unselected IVMF zygotes to the blastocyst stage in vitro.

INTRODUCTION

The development of specific culture regimes capable of supporting in vitro maturation (IVM) and in vitro fertilization (IVF) of oocytes and subsequent development to the blastocyst stage has continued unabated in recent years; it is now possible to obtain preimplantation embryos of all stages from bovine [1–6], ovine [7–13], and porcine [14–16] oocyte-cumulus complexes (OCC) that have been matured, fertilized, and cultured in vitro (IVMFC). Progress has occurred predominantly through the implementation of at least three experimental approaches. In the first approach, complex media such as TCM-199 supplemented with serum are employed in combination with embryo coculture (often on primary oviduct epithelial cell monolayers) in an effort to support embryonic development through the sensitive 8–16-cell stage of bovine or ovine preimplantation development [17–24]. The second approach has resulted from the revitalization of simple defined salt solutions (e.g., synthetic oviduct fluid medium [SOFM]) supplemented with a serum source (such as human serum [HS]) [25–27]. The third and most recently applied approach involves investigating embryonic development in chemically defined protein-free media such as hamster embryo culture media (HECM) [2] or CZB [13, 28–29]. Variations in this design have included the supplementation of serum-free media with synthetic serum substitutes [12] and the addition of specific growth factors [30–31]. The progress with this latter approach has been encouraging, and it seems likely that it will eventually result in the characterization of specific defined culture environments for the development of bovine, ovine, and porcine embryos.

We have continued to explore the mechanisms that underlie the successful development of ovine and bovine preimplantation embryos in vitro. The aim of the present study was to directly compare, for the first time, the effectiveness of two distinct methods of supporting ovine preimplantation development in vitro by investigating the roles of medium composition, serum source and concentration, embryo coculture on primary oviduct epithelial cells, and embryo culture under a reduced O_2 atmosphere.

Accepted November 8, 1993.
Received July 14, 1993.
[1]This research was supported by grants from the Meat Research Corporation of Australia to R.F.S. and D.T.A. Travel support to A.J.W. was provided by a Medical Research Council (MRC) of Canada Visiting Scientist Travel Award.
[2]Correspondence: Dr. Andrew J. Watson, Department of Obstetrics and Gynaecology, University of Western Ontario, University Hospital, 339 Windermere Road, London, Ontario, Canada N6A 5A5. FAX: (519) 663-3388.

MATERIALS AND METHODS

IVM and IVF of Ovine OCC

Up to 200–300 ovaries were collected on a daily basis from a local slaughterhouse and were transported to the laboratory (at room temperature) in PBS containing 100 IU/ml penicillin and 100 μg/ml streptomycin. Ovarian follicles (2–8 mm) were aspirated within 4 h of removal from the animal by means of an 18-gauge needle connected to a 10-ml vacutainer containing 2 ml of oocyte collection medium. This medium consisted of Hepes-buffered TCM-199 medium (Gibco, Grand Island, NY) + 2% (v/v) fetal bovine serum (FBS; Gibco) and 100 IU/ml heparin (Sigma, St. Louis, MO). The OCC were pooled and washed a minimum of six times with collection medium before being placed into maturation medium TCM-199 (Gibco) containing 10% (v/v) FBS (Gibco) supplemented with sodium pyruvate (35 μg/ml; Sigma), 5 μg/ml FSH (Folltropin; Vetrepharm, London, ON, Canada), 5 μg/ml LH (Vetrepharm), and 1 μg/ml estradiol-17β (Sigma) for 24 h at 38.6°C in a humidified, 5% CO_2 atmosphere in air [1]. No selection of OCC was performed at any point during the application of the ovine embryo culture procedures.

For each insemination, four frozen pellets representing a minimum of two rams were thawed at 37°C for 1 min and then processed for normal "swim-up" procedures by layering the semen (0.4 ml) under 1 ml of warmed (38.6°C) Hepes-buffered modified Tyrode's salt solution [32] (without BSA), supplemented with 2% (v/v) heat-inactivated sheep serum, for 2 h at 38.6°C. The OCC were removed from the oocyte maturation medium and subjected to a minimum of four washes through 100-μl drops of Hepes-buffered modified Tyrode's solution (without BSA) supplemented with 2% (v/v) heat-inactivated sheep serum approximately 1/2 h before addition of the motile sperm fraction. The washed oocytes were then allocated to pre-equilibrated fertilization drops (50 OCC/300-μl drop) consisting of modified bicarbonate-buffered Tyrode's salt solution [32] (without BSA or heparin) supplemented with 2% (v/v) sheep serum under light paraffin oil (BDH Inc., Toronto, ON, Canada). After "swim-up," the upper motile sperm fraction was removed (500 μl) and the samples were counted prior to the introduction of approximately 50 μl (or 50–75 × 10^3 motile spermatozoa) of sperm solution per fertilization drop. The sperm/OCC droplets were incubated for 24 h at 38.6°C under an atmosphere of 5% CO_2 in air before removal of the remaining cumulus cell investment. This was followed by introduction of the fertilized zygotes into the appropriate culture treatment group.

Oviduct Cell Monolayer Formation

The primary ovine oviduct epithelial cell monolayers were established as described for bovine oviduct cell monolayers [1]. Oviducts were collected randomly at a local abattoir and were placed in small plastic jars containing 50 ml of Hanks' balanced salt solution (HBSS; Gibco) supplemented with 250 μg/ml each of kanamycin sulfate and gentamycin sulfate (both Sigma) at room temperature. The oviducts were trimmed of connective tissue and rinsed in HBSS + antibiotics before the lumen of each oviduct was filled with approximately 1–2 ml of a 0.05% trypsin-HBSS solution (Gibco). One end of the oviduct was clamped with a hemostat, the trypsin solution was added until the oviduct became rigid, and the open end was clamped. The oviducts were incubated at 38.6°C for 20 min before the contents of the lumen were squeezed into a small Petri dish (35 mm) with a pair of forceps. The cell clumps were further disrupted by forcing the cell preparation through an 18-gauge needle three times. After dispersal, 1 ml of FBS was added to each dish to inhibit further enzymatic digestion and the cell preparation was transferred to 10-ml snap-cap centrifuge tubes (Gibco). The cells were washed four times by addition of 10 ml of HBSS; this was followed by dispersion of the pellet and centrifugation at 500 rpm for 10 min. After the last wash, the pellet was resuspended in TCM-199 + 10% (v/v) FBS; the cells were counted and then plated into 24-well culture plates containing 1 ml of TCM-199 + 10% FBS/well at an approximate density of 1 × 10^6 cells per well.

Embryo Coculture and Microdrop Culture Methods

The oviduct cultures were established two days prior to their use for embryo coculture [1]. This time was sufficient to initiate monolayer formation resulting in a 50–60% confluent cell monolayer. At this time, each well was washed twice with HBSS (1 ml/well) to remove the unattached cells and cell clumps, leaving behind the attached monolayer. One milliliter of fresh embryo culture medium was added to each washed well. Depending upon the experiment (see below), the embryo culture medium was one of the following: 1) TCM-199 + either 10% FBS (Gibco) or 10% HS; 2) bicarbonate-buffered glucose-free synthetic oviduct fluid medium (mod-SOFM) + either 10% FBS or 10% HS; or 3) bicarbonate-buffered modified Tyrode's medium (mod-TALP) [32] without BSA or heparin, supplemented with 10% FBS. The HS was prepared by collecting 80 ml of whole blood from 24-h-fasted subjects. Clot formation was allowed to occur at room temperature for 4 h, and the samples were centrifuged to contract the clots and allow for the collection of up to 40 ml of serum. The serum was heat inactivated at 56.0°C for 30 min, filtered through a 0.22-μm Millipore (Bedford, MA) filter unit, and then stored at −20°C until used. The same HS preparation was utilized for all of the experimental replicates presented in this paper. The cells and fresh media were equilibrated under the appropriate gas atmosphere (see below) at 38.6°C for 2 h before the addition of up to 35 zygotes per well. Embryo coculture was maintained for a maximum of 8 days. The cocultured embryos were moved to fresh 48-h epithelial cell cultures every 48 h.

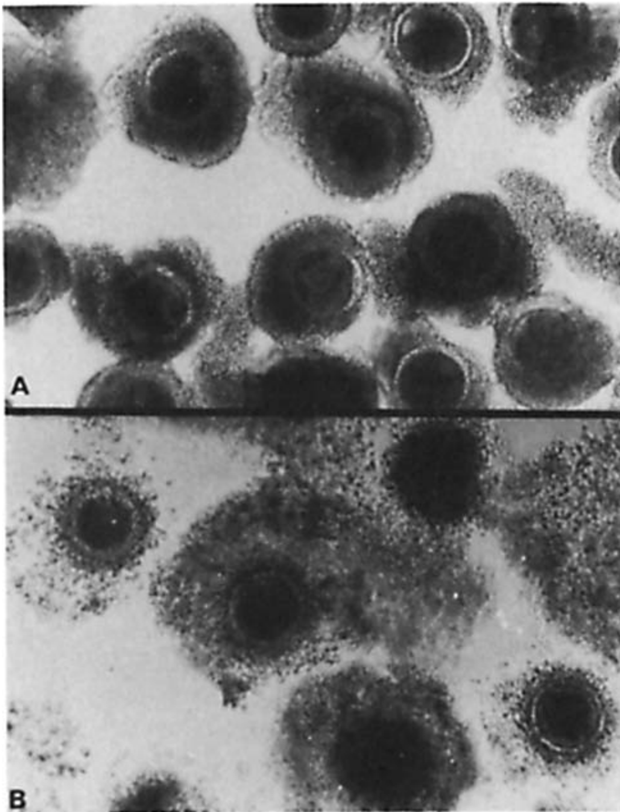

FIG. 1. Morphology of ovine OCC before (A) and after IVM for 24 h (B). The OCC were not uniform, as variation in the extent of cumulus cell investment and in the pattern of oocyte pigmentation was observed within each OCC pool (A). However, no selection criteria were applied, and all of the collected OCC were matured and fertilized in vitro and placed into one of the embryo culture treatments. The cumulus was observed to undergo a dramatic FSH-mediated expansion after 24 h of IVM (B). Scale bars = 120 µm.

Non-cocultured zygotes were maintained in 25-µl drops of embryo culture medium (the same three media outlined above). The culture drops were covered with light paraffin oil and pre-equilibrated either in a 5% CO_2 atmosphere in air or in a glass desiccator jar in which a 5% CO_2:5% O_2:90% N_2 gas mixture continuously flowed. This entire unit was housed within a CO_2 incubator maintained at 38.6°C under a 5% CO_2 atmosphere in air. Up to 10 zygotes were placed in each equilibrated 25-µl culture drop and maintained under the appropriate culture atmosphere for the 8-day culture interval.

Oocyte Maturation and Blastocyst Cell Number

Before the start of the culture experiments, a random sample of 584 IVM oocytes were fixed in ethanol:acetic acid (3:1) for 24 h; they were then stained with 1% aceto-orcein stain to visualize meiotic chromosomal spreads and thus determine the state of meiotic maturation for a population of IVM ovine oocytes.

One hundred cocultured and one hundred non-cocultured Day 8 blastocysts (chosen randomly from the three experiments) were incubated with the fluorescent nuclear stain 4′,6-diamidine-2-phenylindole (DAPI; 1 µg/ml in PBS; Sigma) for 15 min to determine blastocyst cell numbers [1]. The DAPI-stained blastocysts were viewed with a Nikon photomicroscope equipped with epifluorescence optics.

Experiment 1

The aim of experiment 1 was to determine the proportion of IVMF zygotes capable of developing into blastocysts under oviduct cell coculture conditions and also to investigate whether this development was dependent upon the use of complex media such as TCM-199. The experiment was designed as a 2 × 3 factorial to examine the effect of two culture systems (coculture vs. non-coculture) and three culture media (TCM-199 + 10% FBS, mod-SOFM + 10% FBS, and mod-TALP medium + 10% FBS). Zygotes representing four replicates (978 zygotes in total) were randomly assigned to each of the six experimental treatments (163 per treatment). The embryos were maintained in culture for 8 days at 38.6°C under a 5% CO_2 atmosphere in air. The zygotes were evaluated at 24 h after insemination (just prior to their introduction to embryo culture medium) and then at 3, 6, and 8 days after insemination.

Experiment 2

A 2 × 2 × 2 factorial design was used to investigate the roles of embryo culture (coculture vs. non-coculture), medium (TCM-199 vs. mod-SOFM), and serum source (10% FBS vs. 10% HS) upon the development of IVMF zygotes. Zygotes representing three experimental replicates (888 zygotes in total) were randomly assigned to each of the eight experimental treatments (111 per treatment). Development was assessed at 24 h, 72 h, 6 days, and 8 days after insemination. The culture environment was maintained at 38.6°C under a 5% CO_2 atmosphere in air.

Experiment 3

The design of a third experiment was identical to that of experiment 2 except that embryo development was assessed under a low O_2 atmosphere (5% CO_2:5% O_2:90% N_2). A total of 130 IVMF ovine zygotes representing four experimental replicates (1040 zygotes in total) were randomly assigned to each of the eight experimental treatments. The zygotes were evaluated at 24 h, 72 h, 6 days, and 8 days after insemination.

Statistical Analysis

The results were analyzed by step-wise (in reverse) polychotomous logistic regression [33] in the BMDP statistical package to determine the influence of embryo coculture with oviduct epithelial cell monolayers, medium composition, and serum source on the proportion of zygotes ca-

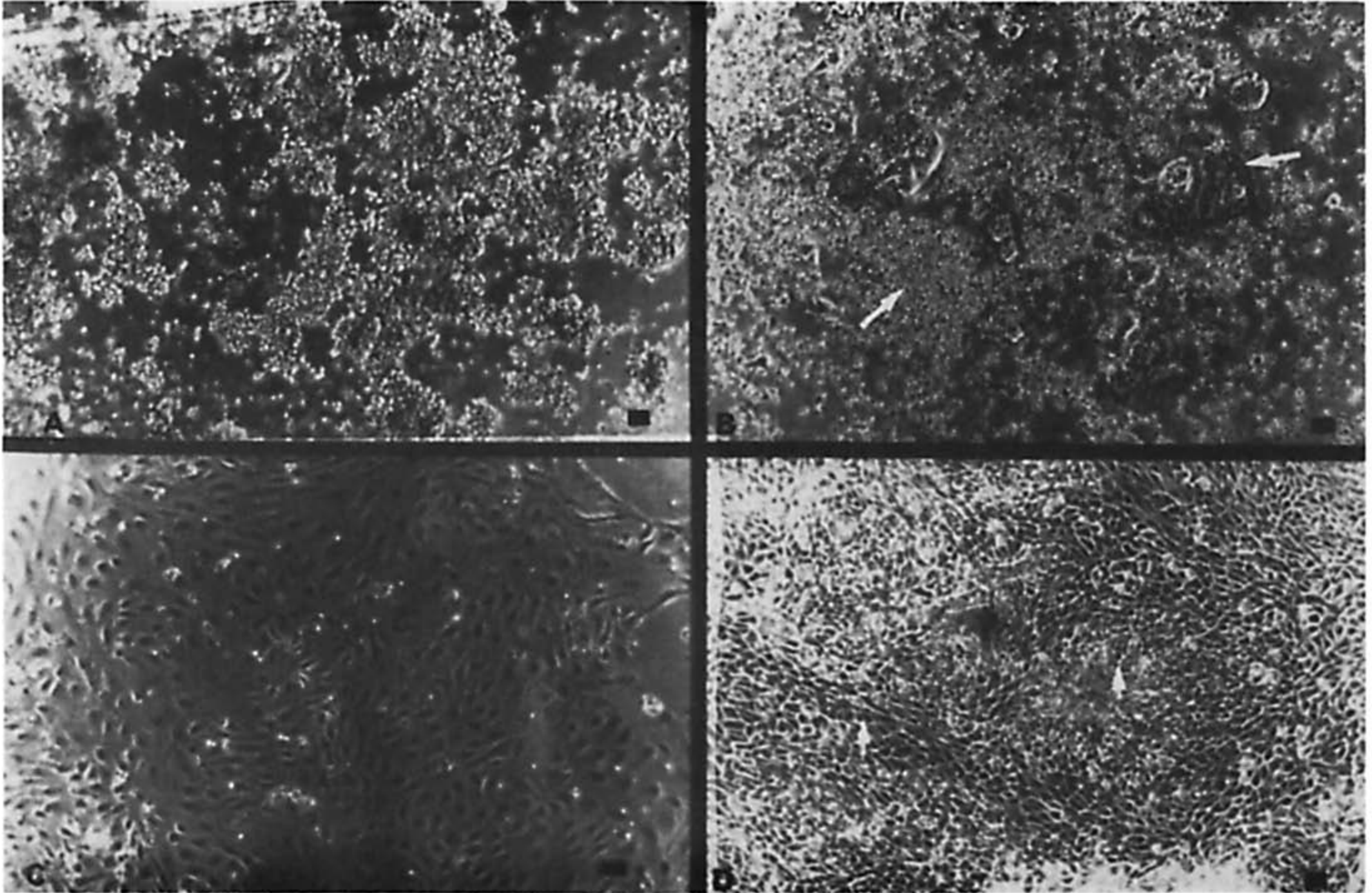

FIG. 2. Primary ovine epithelial cell cultures were initially composed of dispersed single cells and small ciliated cell clumps (A). After 24 h in culture (B), these cells proliferated and assumed three distinct morphologies: 1) nonattached single cells (arrowhead), 2) nonattached ciliated vesicles, and 3) attached and spreading islands of cells (arrowhead). By 48 h in culture, the attached and spreading cells covered up to 50–60% of the surface of the culture well (C). The nonattached cells were removed, leaving behind the attached monolayer that was ready to support embryo coculture. By 72 h, the monolayer was confluent and consisted of at least two cell populations (D). One was composed of islands of cuboidal ciliated cells (arrowhead), which were separated by the second population of long fusiform cell types (arrowhead, (D). Scale bars = 25 μm.

pable of surviving to the post-insemination times of 72 h, 6 days, and 8 days for each of the experimental treatment groups. Tests of significance were established from the model using chi-square analysis. Tests for interactive effects of coculture, medium source, and serum source were performed first. If no interactions were detected, then the main effects tests were performed. Thus, variables and interactions with p values greater than 0.05 were systematically removed from the model until only the significant ($p < 0.05$) variables remained.

Blastocyst cell number was examined through use of a two-sample t-test analysis [34].

RESULTS

Oocyte Maturation

The OCC utilized in the present study were not subjected to any form of selection at any time during the culture interval. An overall average of 3 OCC were recovered from the medium-sized (2–5 mm) follicles of each ovary. The morphology of the OCC population was reasonably uniform and consistent (Fig. 1A), although variation in the extent of cumulus cell investment surrounding each oocyte and in the oocyte pigmentation was observed (Fig. 1A).

To assess oocyte maturation, 584 IVM OCC were stained before the start of the embryo culture experiments. Of the 584 oocytes, 543 or 92.9% displayed an obvious metaphase II chromosomal spread, suggesting that a 24-h period in maturation medium was sufficient to result in a high frequency of meiotic maturation. Maturation was also assessed by observation of the characteristic FSH-mediated cumulus cell expansion (Fig. 1B).

Primary Oviduct Epithelial Cell Monolayers

The primary oviduct cell cultures were initially composed of dispersed single ciliated and nonciliated cells as

TABLE 1. Influence of oviduct epithelial cell coculture and medium on the development of IVMF ovine zygotes.

Treatment[a]	Zygotes[b]	Cleavage at 72 h	Morulae	Blastocysts
(−) Coculture				
TCM-199	163	121/163 (74.2%)	46 (28.2%)	9 (5.5%)
SOFM	163	135/163 (82.8%)	24 (14.7%)	2 (1.2%)
TALP	163	126/163 (77.3%)	24 (14.7%)	6 (3.7%)
Total	**489**	**382/489 (78.1%)**	**94 (19.2%)**	**17 (3.5%)**
(+) Coculture[c]				
TCM-199	163	135/163 (82.8%)	96 (58.9%)	67 (41.1%)
SOFM	163	134/163 (82.2%)	83 (50.9%)	57 (34.9%)
TALP	163	136/163 (83.4%)	90 (55.2%)	62 (38.0%)
Total	**489**	**405/489 (82.8%)**	**269 (55.0%)**	**186 (38.0%)**

[a]All embryos were cultured in the presence of 10% FBS in a 5% CO_2 in air atmosphere at 38.6°C.
[b]No. of replicates = 4; 24 h post-insemination cleavage = 497/978 (50.8%).
[c]$p < 0.05$, coculture versus non-coculture.

well as small clumps of ciliated epithelial cells (Fig. 2A). During the first 24-h interval, these cells proliferated to assume several morphologies, including ciliated vesicles that were not observed to attach, small dispersed groups of individual cells, and small islands of attached cells that slowly spread over the bottom of the culture wells (Fig. 2B). By 48 h, the cell spreading and attachment had continued until a 50–60% monolayer had formed (Fig. 2C). By 72 h, the monolayers were confluent and were observed to consist of at least two distinct cell types (Fig. 2D). These cell populations consisted of islands of small, cuboidal (often ciliated) cells separated and surrounded by numerous tracts of slender fusiform cells (Fig. 2D).

Experiment 1

The method of culture represented the only factor that significantly influenced ($p < 0.05$) embryonic survivability, with greater numbers of zygotes progressing to the morula and blastocyst stages in the coculture treatments compared with the non-coculture treatments (Table 1). A high cleavage frequency (74.2–83.4%) was observed in all of the culture treatments. Although a larger proportion of the cocultured zygotes progressed to the morula and blastocyst stages in the TCM-199 medium treatment compared to the mod-SOFM and mod-TALP medium treatments, no significant differences between these treatments was observed.

Experiment 2

Both embryo culture system and culture medium displayed an independent but significant ($p < 0.05$) influence on the proportion of zygotes that developed to the blastocyst stage (Table 2). The serum source (FBS vs. HS) was not observed to exert a significant influence. Significantly higher ($p < 0.05$) frequencies of cocultured zygotes, com-

TABLE 2. Influence of serum on the development of IVMF ovine zygotes.

Treatment[a]	Zygotes[b]	Cleavage at 72 h	Morulae	Blastocysts
(−) Coculture				
FBS	111	89/111 (80.2%)	9 (8.1%)	2 (1.8%)
TCM-199[d]				
HS	111	90/111 (81.1%)	21 (18.9%)	9 (8.1%)
FBS	111	85/111 (76.6%)	10 (9.0%)	1 (0.9%)
SOFM				
HS	111	80/111 (72.1%)	12 (10.8%)	6 (5.4%)
Total	**444**	**344/444 (77.5%)**	**52 (11.7%)**	**18 (4.0%)**
(+) Coculture[c]				
FBS	111	88/111 (79.3%)	56 (50.5%)	41 (36.9%)
TCM-199				
HS	111	86/111 (77.5%)	55 (49.5%)	42 (37.8%)
FBS	111	91/111 (81.9%)	43 (38.7%)	29 (26.1%)
SOFM				
HS	111	86/111 (77.5%)	41 (36.9%)	23 (20.7%)
Total	**444**	**351/444 (79.0%)**	**195 (43.9%)**	**135 (30.4%)**

[a]All embryos were cultured in the presence of 10% serum in a 5% CO_2 in air atmosphere at 38.6°C.
[b]No. of replicates = 3; 24 h post-insemination cleavage = 397/888 (44.7%).
[c]$p < 0.05$, coculture versus non-coculture.
[d]$p < 0.05$, TCM-199 medium versus mod-SOFM.

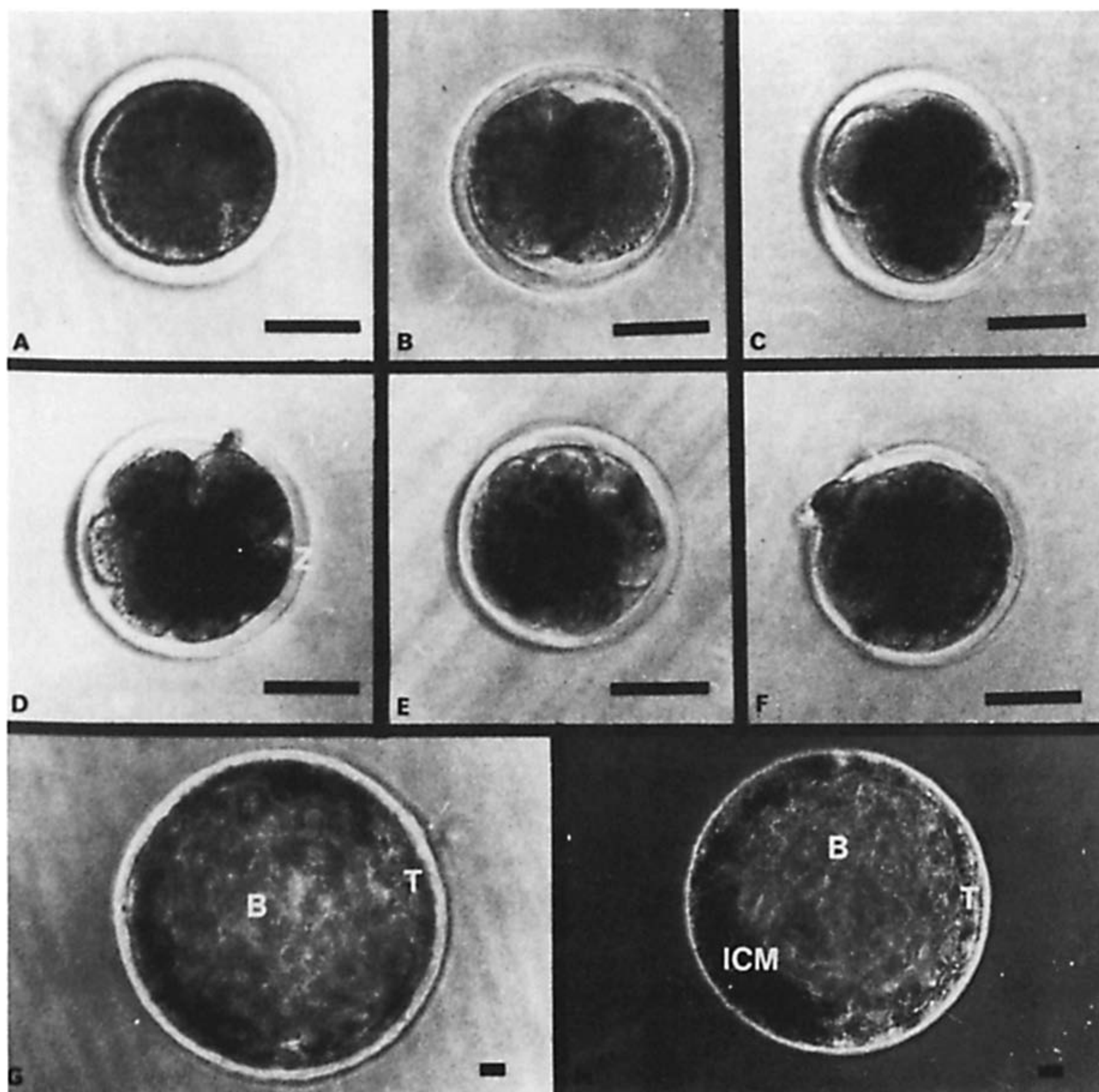

FIG. 3. Morphology of IVMFC ovine preimplantation embryos. Phase-contrast micrographs of (A) 1-cell zygote, (B) 2-cell embryo, (C) 4-cell embryo, (D) 8-cell embryo, (E) 8–16-cell embryo, (F) morula stage (> 30 cells), (G) non-cocultured blastocyst, and (H) cocultured blastocyst. T, trophectoderm; ICM, inner cell mass; B, blastocoel cavity; Z, zona pellucida. Scale bars = 60 μm.

pared to non-cocultured zygotes, developed to the morula and blastocyst stages. A significant difference ($p < 0.05$) in the proportions of zygotes surviving to the morula and blastocyst stages was observed between the TCM-199 and the mod-SOFM treatments for the cocultured and non-cocultured treatment groups. This result suggests that for embryo culture, TCM-199 medium may be preferable to mod-SOFM when culture occurs under a 5% CO_2 atmosphere in air.

Experiment 3

The influence of culture under a reduced O_2 atmosphere on the survival rates of IVMF ovine zygotes through to the morula and blastocyst stages was next investigated by culturing all zygotes in the eight treatment groups under a 5% CO_2:5% O_2:90% N_2 atmosphere. Under these culture conditions, the survival rate of IVMF zygotes to the morula and blastocyst stages was significantly higher ($p < 0.05$) in the non-cocultured treatment groups compared with the coculture groups. Furthermore, serum source also significantly ($p < 0.05$) influenced development to the morula and blastocyst stages. This effect was revealed by significantly higher frequencies of development ($p < 0.05$) to the blastocyst stage in both the TCM-199 and mod-SOFM media supplemented with 10% HS compared to the frequencies for zygotes cultured in the same media supplemented with

TABLE 3. Development of IVMF ovine zygotes under a reduced oxygen culture atmosphere.

Treatment[a]	Zygotes[b]	Cleavage at 72 h	Morulae	Blastocysts
(−) Coculture[c]				
FBS	130	106/130 (81.5%)	49 (37.7%)	8 (6.2%)
TCM-199				
HS[d]	130	103/130 (79.2%)	71 (54.6%)	42 (32.3%)
FBS	130	100/130 (76.9%)	48 (36.9%)	22 (16.9%)
SOFM				
HS	130	103/130 (79.2%)	60 (46.2%)	42 (32.3%)
Total	**520**	**412/520 (79.2%)**	**228 (43.8%)**	**114 (21.9%)**
(+) Coculture				
FBS	130	97/130 (74.6%)	28 (21.5%)	2 (1.5%)
TCM-199				
HS	130	94/130 (72.3%)	25 (19.2%)	0 (0%)
FBS	130	97/130 (74.6%)	5 (3.8%)	0 (0%)
SOFM				
HS	130	77/130 (59.2%)	10 (7.7%)	0 (0%)
Total	**520**	**365/520 (70.1%)**	**68 (13.1%)**	**2 (0.38%)**

[a]All embryos were cultured in the presence of 10% serum in a low oxygen (5% CO_2:5% O_2:90% N_2) atmosphere at 38.6°C.
[b]No. of replicates = 4; 24 h post-insemination cleavage = 387/1040 (37.2%).
[c]$p < 0.05$, non-coculture versus coculture.
[d]$p < 0.05$, HS versus FBS.

10% FBS. These results suggest, in contrast to the initial experiments, that significantly higher ($p < 0.05$) IVMF zygote survival rates are observed when non-coculture in medium supplemented with 10% HS is employed in combination with a reduced oxygen (5% CO_2:5% O_2:90% N_2) culture atmosphere.

Ovine Preimplantation Embryos and Blastocyst Cell Numbers

The morphology of a typical developmental series of IVMFC ovine zygotes is displayed in Figure 3, A-H. No obvious difference was observed in the morphology of non-cocultured (Fig. 3G) vs. cocultured (Fig. 3H) blastocysts. Cell number counts were conducted on each of 100 DAPI-stained non-cocultured and cocultured IVMF blastocysts (Table 4). No significant difference was observed in cell number between these two treatment groups.

DISCUSSION

In the current study we have investigated the influence of embryo coculture, medium composition, serum source, and culture under reduced O_2 levels on the development of IVMFC zygotes to the blastocyst stage. Under the culture atmosphere of 5% CO_2 in air, coculture on ovine primary oviduct epithelial cell monolayers was clearly superior to non-coculture procedures. No influence of serum source was detected under these culture conditions; but composition of the medium was important, since the results suggested that higher proportions of IVMF zygotes survive to the blastocyst stage with use of TCM-199 than with use of glucose-free SOFM.

The optimal culture conditions changed dramatically in experiment 3, in which zygotes were exposed to a 5% CO_2:5% O_2:90% N_2 atmosphere. In this case, embryo survival to the blastocyst stage was greater in the non-cocultured treatments.

The results have thus uncovered two distinct, but effective, culture systems capable of providing a suitable environment for high rates of survival of IVMF zygotes through to the blastocyst stage. The first method involves embryo coculture on primary ovine oviduct epithelial cell monolayers in TCM-199 medium supplemented with either 10% FBS or 10% HS under a 5% CO_2 atmosphere in air; the second approach involves embryo culture in glucose-free SOFM supplemented with 10% HS under a 5% CO_2:5% O_2:90% N_2 atmosphere.

TABLE 4. Cell number comparison of oviduct cell cocultured and non-cocultured blastocysts.

Treatment	Blastocysts[a]	Cell number/blastocyst[b]
Oviduct cell Coculture in TCM-199 +10% FBS and 5% CO_2 in air at 38.6°C	100	77.43 ± 3.19
Non-coculture in mod-SOFM + 10% HS and 5% CO_2:5% O_2:90% N_2 at 38.6°C	100	72.85 ± 3.47

[a]All blastocysts were removed from culture for analysis 8 days post-insemination.
[4b]No significant difference in cell number was observed; values shown are the mean ± SEM.

Embryo coculture on primary oviduct epithelial cell cultures has emerged as an important method capable of supporting development of high proportions of in vivo and in vitro bovine zygotes through the preimplantation interval to the blastocyst stage [1, 17, 20–24, 35]. This beneficial effect of coculture is observed for other species, particularly for in vivo-matured/fertilized ovine zygotes [19]. We have confirmed in the present study that the positive influence of embryo coculture on cell monolayers is extended to IVMF ovine zygotes. Coculture may exert a positive influence on early development by the secretion of "embryotrophic" factors into the culture medium and/or by reducing the negative effects of "toxic" components of the culture environment on preimplantation development [35]. It seems likely that embryo coculture is effective by both of these routes. A beneficial effect on support of the early development of IVMF bovine zygotes to the blastocyst stage is observed by coculture on a variety of diverse cell types including bovine granulosa, oviduct, and uterine cell monolayers, chicken skin cell monolayers, liver cell monolayers, and mouse testicular cell monolayers [5]. This observation suggests that at least part of the coculture influence on early development is a very general one that could reflect secretion into the medium of a pool of "embryotrophic" factors that are not cell-type specific, or might simply consist of a common ability of these cell types to purify the culture environment by acting as a sink for the removal of deleterious culture elements. However, there is a definite cell type-specific influence superimposed upon this more general effect, as primary oviduct epithelial cell cultures are capable of exerting an additional beneficial influence on the development of in vivo-matured/fertilized ovine zygotes through to the blastocyst stage over that observed for fetal sheep fibroblast cell lines (42% blastocysts vs. 4.5%) [19].

For these reasons, a great deal of attention has focused upon the oviduct and its apparent role in supporting preimplantation embryonic development. Oviductal secretory proteins have now been isolated within the human [36], baboon [37], pig [38], cow [39, 40], rabbit [41], and sheep [42–44] oviduct. In sheep, an estrus-associated 90–92-kDa oviductal glycoprotein, capable of binding to the zona pellucida and blastomere surface, has been characterized; it has also been localized within the perivitelline space of early zygotes [42–44]. In addition, the mRNA transcripts for several growth factor ligands—including insulin-like growth factor (IGF)-I, IGF-II, transforming growth factor α (TGFα), basic fibroblast growth factor (bFGF), and TGFβ—have been detected by reverse-transcription polymerase chain reaction (RT-PCR) within 48-h primary bovine and ovine oviduct epithelial cell monolayers [45–46]. The precise role for these molecules in supporting early ovine and bovine development is unclear; but there is evidence that up to 38.8% of bovine IVMF zygotes can progress beyond the 16-cell stage, with 24.6% reaching the blastocyst stage [30], in media supplemented with bFGF and TGFβ, suggesting that growth factors could certainly perform roles expected for "embryotrophic" factors. These molecules are therefore good candidates for further experimentation directed at clarifying the molecular nature of the beneficial coculture influence on early mammalian preimplantation development.

Is it possible that the oviductal cells are performing an additional role? Tervit et al. [25] first reported the benefit of culturing ovine and bovine in vivo-matured/fertilized zygotes in a simple defined salt solution under a reduced oxygen environment of 5% CO_2:5% O_2:90% N_2. This approach has been recently revitalized and improved upon by the addition of 20% HS to the culture medium, resulting in up to 80% of ovine zygotes progressing through to the blastocyst stage [7–9, 47]. Similar benefits of culturing preimplantation goat and mouse embryos under a reduced oxygen environment have been observed, suggesting that it may be important to limit the exposure time of zygotes to atmospheric oxygen in order to obtain optimal development through the preimplantation interval [48–52].

All mammalian cells are vulnerable to damage by active oxygen that is a byproduct of cellular oxidative/reduction reactions [53, 54]. Superoxide anions and hydrogen peroxide can react with polypeptides and membrane lipids, resulting in cell damage by enzyme inactivation and lipid peroxidation [53, 54]. This cellular damage could be offset, in part, by the dismutation of superoxide free radicals catalyzed by superoxide dismutase (SOD) [53, 54]. SOD activity has been measured within rabbit oviductal fluid, supporting a role for the oviduct in minimizing the detrimental effects of free O_2-radical formation on preimplantation embryonic development [51]. Our results suggest that this is a probable component of the overall beneficial influence of oviductal cell coculture on embryo development under an atmosphere of 5% CO_2 in air. Oviductal cell coculture was, however, not beneficial under a 5% CO_2:5% O_2:90% N_2 atmosphere, presumably because this reduced O_2 concentration was unable to maintain oviductal cell viability.

A direct role for the production of oxygen free radicals and hydrogen peroxide in contributing to the onset of culture blocks in mammalian preimplantation development must still be established. Hydrogen peroxide levels are observed to increase within 1- to 2-cell cultured mouse zygotes, but it has been found that a decline in oxygen culture atmosphere from 20% to 5% does not influence rates of development to the blastocyst stage nor the levels of hydrogen peroxide production within cultured mouse zygotes [55–58].

Our results have also clearly established that high rates of early ovine embryo development can be achieved in media supplemented with 10% serum as opposed to the more commonly utilized 20% HS levels [7–10, 26, 47]. Furthermore, the observation that such high frequencies of development to the blastocyst stage can occur with an unselected pool of OCC suggests a contrast with similar efforts in culture of IVMF bovine zygotes, which commonly include sub-

jecting the bovine OCC to an intense selection before or after placement into oocyte maturation medium [1–6, 59, 60].

The precise roles of medium composition, serum source, embryo coculture, and culture atmosphere will be fully elucidated only by further experimentation. However, the characterization of two distinct, but equally effective, culture environments that are capable of supporting the development of high proportions of IVMF ovine zygotes through to the blastocyst stage should expedite our understanding of the optimal in vitro environments required to sustain mammalian preimplantation embryo development.

ACKNOWLEDGMENTS

Appreciation is extended to Vicky Tregenza and Jane Lang for assistance with the ovine embryo culture procedures and to Ms. Barbara Lowery for preparing the final manuscript.

REFERENCES

1. Wiemer KE, Watson AJ, Polanski V, McKenna AI, Fick GH, Schultz GA. Effects of maturation and coculture treatments on the developmental capacity of early bovine embryos. Mol Reprod Dev 1991; 30:330–338.
2. Pinyopummintr T, Bavister BD. In vitro-matured/in vitro fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined, protein-free culture media. Biol Reprod 1991; 45:736–742.
3. Sirard MA, Parrish JJ, Ware ML, Leibfried-Rutledge ML, First NL. The culture of bovine oocytes to obtain developmentally competent embryos. Biol Reprod 1988; 39:546–552.
4. Fukui Y, Ono H. Effects of sera, hormones and granulosa cells added to culture medium for in-vitro maturation, fertilization, cleavage and development of bovine oocytes. J Reprod Fertil 1989; 86:501–506.
5. Goto K, Kajihara Y, Kosaka S, Koba M, Nakanishi Y, Ogawa K. Pregnancies after coculture of cumulus cells with bovine embryos derived from in-vitro fertilization of in-vitro matured follicular oocytes. J Reprod Fertil 1988; 83:753–758.
6. Fukuda Y, Ichikawa M, Naito K, Toyoda Y. Birth of normal calves resulting from bovine oocytes matured, fertilized and cultured with cumulus cells in vitro up to the blastocyst stage. Biol Reprod 1990; 42:114–119.
7. Walker SK, Lampe RJ, Seamark RF. Culture of sheep zygotes in synthetic oviduct fluid medium with different concentrations of sodium bicarbonate and hepes. Theriogenology 1989; 32:797–804.
8. Thompson JGE, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. J Reprod Fertil 1990; 89:573–578.
9. Thompson JG, Simpson AC, Pugh PA, Tervit HR. Requirement for glucose during in vitro culture of sheep preimplantation embryos. Mol Reprod Dev 1992; 31:253–257.
10. Pugh PA, Fukui Y, Tervit HR, Thompson JG. Developmental ability of in vitro matured sheep oocytes collected during the nonbreeding season and fertilized in vitro with frozen ram semen. Theriogenology 1991; 36:771–778.
11. Czlonkowska M, Eysymont U, Guszkiewicz A, Kossakowski M, Dzizk J. Birth of lambs after in vitro maturation, fertilization and coculture with oviductal cells. Mol Reprod Dev 1991; 30:34–38.
12. Russeler-Long JA, Dickey JF, Richardson ME, Ivey KW. Culture of ovine embryos in the absence of bovine serum albumin. Theriogenology 1991; 35:383–391.
13. McGinnis LK, Youngs CR. In vitro development of ovine embryos in CZB medium. Theriogenology 1992; 37:559–569.
14. Yoshida M, Ishizaki Y, Kawagishi H. Blastocyst formation by pig embryos resulting from in-vitro fertilization of oocytes matured in vitro. J Reprod Fertil 1990; 88:1–8.
15. Rath D. Experiments to improve in vitro fertilization techniques for in vivo-matured porcine oocytes. Theriogenology 1992; 37:885–896.
16. Zheng YS, Sirard MA. The effect of sera, bovine serum albumin and follicular cells on in vitro maturation and fertilization of porcine oocytes. Theriogenology 1992; 37:779–790.
17. Eyestone WH, First NL. Coculture of early cattle embryos to the blastocyst stage with oviducal tissue or in conditioned medium. J Reprod Fertil 1989; 85:715–720.
18. Sparks AET, Gwazdauskas FC, McGilliard ML. Culture of one-cell bovine embryos in explanted mouse oviduct and bovine epithelial cells. Theriogenology 1992; 37:587–594.
19. Gandolfi F, Moor RM. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J Reprod Fertil 1987; 81:23–28.
20. Goto K, Iwai Y, Takuma Y, Nakanishi Y. Coculture of in vitro fertilized bovine embryos with different cell monolayers. J Anim Sci 1992; 70:1449–1453.
21. Takeuchi K, Nagata Y, Sanow BA, Hodgen GD. Primary culture of human fallopian tube epithelial cells and co-culture of early mouse pre-embryos. Mol Reprod Dev 1992; 32:236–242.
22. Ellington JE, Carney EW, Farrell PB, Simkin ME, Foote RH. Bovine 1–2-cell embryo development using a simple medium in three oviduct epithelial cell coculture systems. Biol Reprod 1990; 43:97–104.
23. Eyestone WH, Jones JM, First NL. Some factors affecting the efficacy of oviduct tissue-conditioned medium for the culture of early bovine embryos. J Reprod Fertil 1991; 92:59–64.
24. Xu KP, Yadav BR, Rorie RW, Plante L, Betteridge KJ, King WA. Development and viability of bovine embryos derived from oocytes matured and fertilized in vitro and co-cultured with bovine oviducal epithelial cells. J Reprod Fertil 1992; 94:33–43.
25. Tervit HR, Whittingham DG, Rowson LEA. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 1972; 30:493–497.
26. McLaughlin KJ, McLean DM, Stevens G, Ashman RA, Lewis PA, Bartsch BD, Seamark RF. Viability of one-cell bovine embryos cultured in synthetic oviduct fluid medium. Theriogenology 1990; 33:1191–1199.
27. Fukui Y, McGowan LT, James RW, Pugh PA, Tervit HR. Factors affecting the in vitro development to blastocysts of bovine oocytes matured and fertilized in vitro. J Reprod Fertil 1991; 92:125–131.
28. Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J Reprod Fertil 1989; 86:679–688.
29. Chatot CL, Lewis JL, Torres I, Ziomek CA. Development of 1-cell embryos from different strains of mice in CZB medium. Biol Reprod 1990; 42:432–440.
30. Larson RC, Ignotz GG, Currie WB. Transforming growth factor β and basic fibroblast growth factor synergistically promote early bovine embryo development during the fourth cell cycle. Mol Reprod Dev 1992; 33:432–435.
31. Larson RC, Ignotz GG, Currie WB. Platelet derived growth factor (PDGF) stimulates development of bovine embryos during the fourth cell cycle. Development 1992; 115:821–826.
32. Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Crister ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen thawed sperm. Theriogenology 1986; 25:591–600.
33. McCullagh P. Regression models for ordinal data. J R Statist Soc B 1980; 42:109–142.
34. Mathews DE, Farewell VT. Using and Understanding Medical Statistics, 2nd ed. New York: Karger; 1988: 288.
35. Bavister BD. Role of oviductal secretions in embryonic growth in vivo and in vitro. Theriogenology 1988; 29:143–154.
36. Verhage HG, Fazleabas AT, Donnelly K. The in vitro synthesis and release of proteins by the human oviduct. Endocrinology 1988; 122:1639–1645.]
37. Fazleabas AT, Verhage HG. The detection of oviduct specific proteins in the baboon (*Papio anubis*). Endocrinology 1986; 35:455–462.
38. Buhi WC, Alverez IM, Sudhipong V, Dones-Smith MM. Identification and characterization of de novo synthesized porcine oviductal secretory proteins. Biol Reprod 1990; 43:929–938.
39. Boice ML, Geisert RD, Blair RM, Verhage HG. Identification and characterization of bovine oviductal glycoproteins synthesized at estrus. Biol Reprod 1990; 43:457–465.
40. Boice ML, Mavrogianis PA, Murphy CN, Prather RS, Day BN. Immunocytochemical analysis of the association of bovine oviduct-specific glycoproteins with early embryos. J Exp Zool 1992; 263:225–229.
41. Oliphant G, Ross PR. Demonstration of production and isolation of three sulfonated glycoproteins from the rabbit oviduct. Biol Reprod 1982; 26:537–544.
42. Buhi WC, Bazer FW, Alvarez IM, Mirando MA. In vitro synthesis of oviductal proteins associated with estrus and 17-β estradiol treated ovariectomized ewes. Endocrinology 1991; 128:3086–3095.
43. Gandolfi F, Brevini TAL, Richardson L, Brown CR, Moor RM. Characterization of proteins secreted by sheep oviduct epithelial cells and their function in embryonic development. Development 1989; 106:303–312.
44. Murray MK. Biosynthesis and immunocytochemical localization of an estrogen dependent glycoprotein and associated morphological alterations in the sheep ampulla oviduct. Biol Reprod 1992; 47:889–902.

45. Watson AJ, Hogan A, Hahnel A, Wiemer KE, Schultz GA. Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. Mol Reprod Dev 1992; 31:87–95.
46. Watson AJ, Watson PH, Arcellana-Panlilio M, Warnes D, Walker SK, Schultz GA, Armstrong DT, Seamark RF. A growth factor phenotype map for ovine preimplantation development. Biol Reprod 1994; 50:725–733.
47. Walker SK, Seamark RF, Quinn P, Warnes GM, Ashman RJ, Smith DH, Ancell P. Culture of pronuclear embryos of sheep in a simple medium. Proc 11th Int Congr Anim Reprod and AI 1988; 4:483.
48. Batt PA, Gardner DK, Cameron WN. Oxygen concentration and protein source affect the development of preimplantation goat embryo in vitro. Reprod Fertil Dev 1991; 3:601–607.
49. Umaoka Y, Noda Y, Narimoto K, Mori T. Developmental potentiality of embryos cultured under low oxygen tension with superoxide dismutase. J In Vitro Fert Embryo Transfer 1991; 8:245–249.
50. Pabon JE, Findley WE, Gibbons WE. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. Fertil Steril 1989; 51:896–900.
51. Noda Y, Matsumoto H, Umaoka Y, Tatsumi K, Kishi J, Mori T. Involvement of superoxide radicals in the mouse two-cell block. Mol Reprod Dev 1992; 28:356–360.
52. Quinn P, Harlow GM. The effect of oxygen on the development of preimplantation mouse embryos in vitro. J Exp Zool 1978; 206:73–80.
53. Fridovich I. Superoxide radical: an endogenous toxicant. Annu Rev Pharmacol Toxicol 1983; 23:239–257.
54. Halliwell B. Superoxide-dependent formation of hydroxy radicals in presence of iron chelaters. FEBS Lett 1987; 92:321–326.
55. Nasr-Esfahani MH, Aitken JR, Johnson MH. Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed in vitro or in vivo. Development 1990; 109:501–507.
56. Nasr-Esfahani MH, Johnson MH. The origin of reactive oxygen species in mouse embryos cultured in vitro. Development 1991; 113:551–560.
57. Nasr-Esfahani MH, Johnson MH. How does transferrin overcome the in vitro block to development of the mouse preimplantation embryo. J Reprod Fertil 1992; 96:41–48.
58. Nasr-Esfahani MH, Winston NJ, Johnson MH. Effects of glucose, glutamine, ethylenediaminetetraacetic acid and oxygen tension on the concentration of reactive oxygen species and on the development of the mouse preimplantation embryo in vitro. J Reprod Fertil 1992; 96:219–231.
59. Kastrop PMM, Bevers MM, Destree OHJ, Kruip ThAM. Analysis of protein synthesis in morphologically classified follicular oocytes before and after maturation in vitro. Mol Reprod Dev 1990; 26:222–226.
60. Pavlok A, Lucas-Hahn A, Niemann H. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. Mol Reprod Dev 1992; 31:63–67.