Support for the development of bovine embryos *in vitro* by secretions of bovine trophoblastic vesicles derived *in vitro*

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This study evaluated whether trophoblastic tissue derived *in vitro* secretes factors that support bovine embryonic development *in vitro*. The embryotrophic activity of these secretions was analysed in three different culture conditions based on TCM-199: (1) in a routine culture system using cumulus cells and 10% oestrous cow serum; (2) without cells but with 10% oestrous cow serum; and (3) under serum-free conditions. Rates of development to the 5–8-cell and blastocyst stages, as well as numbers of inner cell mass and trophoblast cells of blastocysts were determined. In the absence of cumulus cells, cleavage rates of 5–8-cell embryos were significantly ($P < 0.05$) higher in trophoblastic vesicle-conditioned medium than in TCM-199 in both the presence (71% versus 49%) and absence (70% versus 49%) of serum. Trophoblastic vesicle-conditioned medium had a significant ($P < 0.05$) positive effect on the rate of development to the blastocyst stage when compared with TCM-199 in the presence of cumulus cells and serum (39% versus 33%), only serum (26% versus 19%), or in the absence of cells and serum (21% versus 5%). The numbers of inner cell mass and trophoblast cells, and total number of cells in blastocysts produced in the cumulus cell coculture system in serum-free trophoblastic vesicle-conditioned medium or TCM-199 supplemented with serum were greater than those of blastocysts produced without cumulus cells or serum. Fractionation of serum-free trophoblastic vesicle-conditioned medium by ultrafiltration (10 kDa cut off) confined the embryotrophic activity mainly to the low molecular mass fraction. This study shows that serum-free trophoblastic vesicle-conditioned medium contains potent embryotrophic factors which act in a complementary manner to those secreted by cumulus cells and those supplemented with serum and result in reliably high blastocyst rates in the range of 40%. Since contamination of trophoblastic vesicle-conditioned medium with serum proteins can be avoided, this medium may be a reasonable source for the purification of specific embryotrophic factors.

Introduction

Trophoblastic cells of bovine preimplantation embryos are known to produce a number of factors, including placental lactogen (Flint *et al.*, 1979; Morgan *et al.*, 1989), retinol-binding protein (Liu *et al.*, 1993), pregnancy-associated glycoproteins 1 and 2 (Zoli *et al.*, 1992; Xie *et al.*, 1994), and interferon tau (IFN τ), which is the most important pregnancy recognition signal (Roberts *et al.*, 1992). We have established a culture system in which bovine trophoblastic tissue can be produced entirely *in vitro* and maintained biologically active in terms of IFN τ secretion (Stojkovic *et al.*, 1995a). Trophoblastic cells have been used successfully as a coculture system for the *in vitro* production of bovine embryos (Camous *et al.*, 1984; Stojkovic *et al.*, 1995b). However, specific effects of the trophoblastic cells or their secretions could not be analysed since serum or serum

components were used in the culture media. We noticed that trophoblastic vesicles derived *in vitro* – once established – can survive in serum-free medium. Such media conditioned by trophoblastic vesicles were analysed for their embryotrophic activity. In addition to rates of development to the blastocyst stage, we determined the number of inner cell mass (ICM) and trophoblast (TE) cells of blastocysts. These parameters may be valid indicators of the viability and quality of mammalian blastocysts (Papaioannou and Ebert, 1988).

Materials and Methods

Preparation of trophoblastic vesicles and trophoblastic vesicle-conditioned medium

Trophoblastic tissue was derived *in vitro* as described by Stojkovic *et al.* (1995a). Briefly, day 8 (*in vitro* fertilization =

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day 0) blastocysts produced *in vitro* were transferred individually to 60 µl microdrops of Dulbecco's modified Eagle's medium supplemented with 60% buffalo rat liver (BRL) cell-conditioned Glasgow BHK-21 medium, 10^{-4} mol 2-mercaptoethanol l^{-1} (Merck, Darmstadt) and 15% fetal calf serum (GLCM) at 37°C in an atmosphere of 10% CO₂ in air and maximum humidity. On day 13, the inner cell mass of each blastocyst was removed by microsurgery and the trophoblastic tissue was cultured under the same conditions. After 2 days in culture, isolated trophoblastic tissue had formed spherical trophoblastic vesicles (TVs).

TVs were washed four times in serum-free TCM-199 supplemented with 4.43 mmol Hepes l^{-1} , 33.9 mmol sodium bicarbonate l^{-1} , 2 mmol sodium pyruvate l^{-1} , 2.92 mmol calcium lactate l^{-1} , 60 µg gentamicin ml^{-1} (M-199) and then cultured in this medium at 39°C in an atmosphere of 5% CO₂ and 5% O₂ in air and maximum humidity for 7 days. This trophoblastic vesicle-conditioned medium (TVCM) was collected and frozen at -20°C until use.

In vitro maturation and in vitro fertilization of bovine cumulus-oocyte complexes

Ovaries were collected from a local abattoir and transported to the laboratory in warmed PBS (28°C). Follicles with diameter 2–8 mm were aspirated using a 20 gauge needle and a vacuum pressure of approximately 100 mm Hg. The cumulus-oocyte complexes (COCs) were collected in M-199 supplemented with 10% (v/v) heat-inactivated serum from cows at oestrus (OCS). Only COCs with a complete dense cumulus and a dark, evenly granulated cytoplasm were selected for IVM. The COCs were washed in collecting medium supplemented with 10 µg FSH-p ml^{-1} (FSH-p, Schering Pharmaceuticals, Kenilworth, NJ) and matured for 24 h at 39°C in a atmosphere of 5% CO₂ in air and maximum humidity.

After maturation, the COCs were maintained in a Tyrode's albumin-lactate-pyruvate medium containing 6 mg BSA ml^{-1} , 10 µg heparin ml^{-1} , and frozen-thawed semen (10^6 spermatozoa ml^{-1}) that had been subjected to swim-up procedure (Parrish *et al.*, 1986). COCs were maintained in this medium for 18 h under conditions that had been used for IVM.

In vitro culture of bovine embryos

Cumulus cells were removed from presumptive zygotes by vortexing (120 s) and by gentle pipetting. Zygotes were washed three times in culture media. Groups of 30–35 zygotes were cultured in 400 µl of culture media under paraffin oil (DAB 7160; Merck) at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Culture conditions were: (1) TVCM supplemented with 10% OCS and cumulus cells (TVCM-SC); (2) TVCM supplemented with 10% OCS (TVCM-S); (3) serum-free TVCM (TVCM); (4) M-199 supplemented with 10% OCS and cumulus cells (M-199-SC); (5) M-199 supplemented with 10% OCS (M-199-S); and (6) M-199. The embryotrophic activity of culture media was determined by the

proportion of 5–8-cell embryos and embryos at the blastocyst stage (66 h and 186 h after *in vitro* fertilization, respectively).

Physicochemical treatments of trophoblastic vesicle-conditioned medium

For determination of its physicochemical properties, TVCM was ultrafiltrated through a 10 kDa cut off membrane (Centrisart, Sartorius, Göttingen), frozen and thawed three times, diluted in fresh M-199 boiled (10 min in a 100°C water bath), or decomplexed (30 min in a 56°C water bath) and tested subsequently for embryotrophic activity. The total amount of protein in TVCM was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL).

Differential staining of blastocysts produced in vitro

The ICM and TE nuclei of blastocysts were differentially stained with fluorochromes using a modification of the procedure described by Handyside and Hunter (1984). Briefly, blastocysts were washed several times in Dulbecco's phosphate-buffered saline (PBS) to remove excess protein. Expanded blastocysts were treated with 0.5% (w/v) pronase (Protease, Type XXV, P-6911; Sigma, St Louis, MO) in PBS for 3–5 min to dissolve zonae pellucidae.

Zona-free and hatched blastocysts were incubated in a 1:2 dilution in PBS of antiserum raised in rabbit against *in vitro* produced trophectoderm cell-conditioned medium containing 10% fetal calf serum and 10^4 antiviral units of IFN τ (Stojkovic *et al.*, in press) for 45 min at 39°C in a humidified atmosphere of 5% CO₂. Subsequently, embryos were washed five times in PBS warmed at 39°C and then incubated in PBS supplemented with 5% (v/v) guinea-pig complement (ICN, Immunobiologicals, Costa Mesa, CA) and 50 µg propidium iodide ml^{-1} (P-4170; Sigma) for 45 min at 39°C in a humidified atmosphere of 5% CO₂. This step resulted in the selective antibody-mediated complement lysis of trophectoderm cells (Solter and Knowles, 1975) and the red staining of trophectoderm cell nuclei. After this step, blastocysts were washed again in PBS and then placed in cold absolute ethanol (Merck) containing a fluorochrome: 25 µg bisbenzimidazole ml^{-1} (Riedel-Haen AG, Seelze, Hannover) for 30 min at 4°C. Finally, embryos were washed in absolute ethanol, mounted in undiluted glycerol (Merck) and squashed on a glass slide. The stained embryos were observed using a fluorescent microscope (Axiovert 135, Zeiss, Jena) with an HBO lamp under transmittance illumination and an ultraviolet excitation filter of 365 nm and a barrier filter of 420 nm. Bisbenzimidazole-stained vital ICM nuclei appeared blue, and nonvital TE nuclei, which were stained with both fluorochromes, gave red or pink fluorescence.

Statistical analysis

The proportions of embryos that developed to 5–8-cell and blastocyst stages were analysed by chi-squared test. For comparison of numbers of ICM cells, TE cells, and total number of cells in blastocysts, the Mann-Whitney U test was used.

Table 1. Effects of trophoblastic vesicle-conditioned medium (TVCM) on the development of bovine oocytes 66 h and 186 h after *in vitro* fertilization

Culture	Oocytes	66 h	186 h			Total (%)
		5–8 cells (%)	Blastocyst (%)	Expanded blastocyst (%)	Hatched blastocyst (%)	
TVCM–SC	720	589 (82) ^a	63 (9) ^a	129 (18) ^a	85 (12) ^a	277 (39) ^a
M199–SC	680	528 (78) ^a	45 (7) ^a	95 (14) ^b	82 (12) ^a	222 (33) ^b
TVCM–S	675	478 (71) ^b	38 (6) ^a	95 (14) ^b	43 (6) ^b	176 (26) ^c
M199–S	720	355 (49) ^c	40 (6) ^a	50 (7) ^{cd}	45 (6) ^b	135 (19) ^d
TVCM	720	504 (70) ^b	60 (8) ^a	62 (9) ^c	32 (4) ^b	154 (21) ^d
M199	720	354 (49) ^c	5 (1) ^b	30 (4) ^d	0 (0) ^c	35 (5) ^f

^{a–f}Values with different superscript letters within each column are significantly different (chi-squared test; $P < 0.05$).

TVCM–SC: trophoblastic vesicle-conditioned medium with 10% serum and cumulus cell coculture; M199–SC: modified TCM199 with 10% serum and cumulus cell coculture; TVCM–S: trophoblast vesicle-conditioned medium with 10% serum; M199–S: TCM199 with 10% serum; TVCM: trophoblastic vesicle-conditioned medium; M199: modified TCM 199.

Table 2. Numbers of inner cell mass (ICM) cells, trophoctoderm (TE) cells and total number of cells in blastocysts* cultured in trophoblastic vesicle-conditioned medium (TVCM)

Medium	Number of ICM cells mean $\pm$ SD (range)	Number of TE cells mean $\pm$ SD (range)	Total number of cells mean $\pm$ SD (range)
TVCM–SC	32.2 $\pm$ 5.6 (22–42) ^a	67.5 $\pm$ 9.8 (53–85) ^a	99.7 $\pm$ 13.2 (77–126) ^a
M199–SC	29.9 $\pm$ 6.8 (20–42) ^{ab}	65.1 $\pm$ 15.9 (38–89) ^a	95.0 $\pm$ 19.6 (58–122) ^a
TVCM–S	28.5 $\pm$ 4.5 (21–41) ^b	50.2 $\pm$ 5.6 (41–61) ^b	78.7 $\pm$ 6.1 (63–91) ^b
M199–S	28.2 $\pm$ 5.1 (21–40) ^b	59.9 $\pm$ 9.5 (40–80) ^c	87.7 $\pm$ 12.6 (62–108) ^c
TVCM	24.1 $\pm$ 5.0 (15–32) ^c	45.4 $\pm$ 8.5 (26–60) ^d	69.4 $\pm$ 11.4 (46–88) ^d
M199	19.9 $\pm$ 3.3 (14–26) ^d	45.2 $\pm$ 5.6 (37–57) ^d	65.1 $\pm$ 7.5 (52–77) ^d

*Number of blastocysts examined = 20–22.

^{abcd}Values within the same column with different superscripts are significantly different (Mann–Whitney U test; $P < 0.05$).

TVCM–SC: trophoblastic vesicle-conditioned medium with 10% serum and cumulus cell coculture; M199–SC: modified TCM199 with 10% serum and cumulus cell coculture; TVCM–S: trophoblast vesicle conditioned medium with 10% serum; M199–S: TCM199 with 10% serum; TVCM: trophoblastic vesicle-conditioned medium; M199: modified TCM199.

Results

Effects of trophoblastic vesicle-conditioned medium on development to the 5–8-cell stage

The proportions of inseminated oocytes developing to the 5–8-cell stage under the various culture conditions are shown (Table 1). There was no significant difference between oocytes developed in TVCM and M-199 in terms of the presence of serum and cumulus cells. However, in the absence of cumulus cells, cleavage rates were significantly ($P < 0.05$) higher in TVCM than in M-199, both in the presence and absence of serum.

Effects of trophoblastic vesicle-conditioned medium on development to the blastocyst stage

TVCM had a significant ($P < 0.05$) positive effect on the rate of development to the blastocyst stage compared with M-199 in the presence of cumulus cells and serum, serum alone, or in the absence of cells and serum (Table 1). Coculture with

cumulus cells significantly ($P < 0.05$) increased the proportion of hatched blastocysts in both TVCM and M-199.

Effects of trophoblastic vesicle-conditioned medium on number of cells in blastocysts

The numbers of ICM cells, TE cells and total number of cells in blastocysts produced in the cumulus cell coculture system in TVCM or M-199 supplemented with serum were greater than those of blastocysts produced without cumulus cells or serum (Table 2). A significant ($P < 0.05$) positive effect on the number of ICM cells in TVCM compared with M-199 was observed only in the absence of cumulus cells and serum.

Physicochemical characteristics of trophoblastic vesicle-conditioned medium

The total protein content of undiluted TVCM was 35 $\mu\text{g ml}^{-1}$. Effects of different physicochemical treatments on the embryotrophic activity of TVCM are summarized

Table 3. Effect of physicochemical treatments of trophoblastic vesicle-conditioned medium (TVCM) on *in vitro* development of bovine oocytes after *in vitro* fertilization

Treatment of TVCM	Number of oocytes	Number at 5–8-cell stage (%)	Number of blastocysts (%)
Untreated	720	504 (70) ^a	154 (21) ^a
Ultrafiltration:			
< 10 kDa	600	380 (63) ^b	110 (18) ^{ab}
> 10 kDa	594	231 (39) ^c	50 (8) ^c
Dilution:			
1:2	360	201 (56) ^d	61 (17) ^{ab}
1:4	360	158 (44) ^c	35 (10) ^c
1:24	360	162 (45) ^c	18 (5) ^d
Heat:			
56°C, 30 min	720	476 (66) ^{ab}	116 (16) ^b
100°C, 10 min	720	224 (31) ^c	58 (8) ^c
Freeze–thawing	720	498 (69) ^a	151 (21) ^a

^{abcd}Values within the same column with different superscripts are significantly different (chi-squared test; $P < 0.05$).

Table 4. Numbers of inner cell mass (ICM) cells, trophoctoderm (TE) cells and total number of cells in blastocysts cultured in trophoblastic vesicle-conditioned medium (TVCM) after ultrafiltration

Fraction	ICM cells mean $\pm$ SD (range)	TE cells mean $\pm$ SD (range)	Total cells mean $\pm$ SD (range)
< 10 kDa	26.2 $\pm$ 3.9 (18–33)	53.2 $\pm$ 6.9 (38–60)	79.4 $\pm$ 9.5 (57–91)
> 10 kDa	24.1 $\pm$ 3.1 (19–30)	49.8 $\pm$ 6.4 (35–58)	73.9 $\pm$ 8.6 (59–87)

Number of blastocysts examined = 20.

(Table 3). Fractionation of TVCM by ultrafiltration (10 kDa cut off) confined the embryotrophic activity mainly to the low molecular mass fraction. However, mean number of cells in blastocysts only had a tendency towards difference between the two fractions of TVCM (Table 4). A dose-dependent effect of embryotrophic factors was shown by a decrease in early cleavage and blastocyst rates after gradual dilution of TVCM. Whereas the embryotrophic activity of TVCM was reduced by heat treatment (slightly by 56°C for 30 min, markedly by boiling for 10 min), repeated freezing and thawing of TVCM did not affect its embryotrophic activity.

Discussion

This study evaluated whether trophoblastic tissue derived *in vitro* secretes factors that support bovine embryonic development *in vitro*. The embryotrophic activity of these secretions was analysed in three different culture conditions: (1) in a routine culture system using cumulus cells and 10% serum; (2) without cells but with 10% serum; and (3) under serum-free conditions.

A previous study showed that *in vitro* production and maintenance of trophoblastic vesicles was not successful in M-199 but required more complex culture conditions, such as BRL cell-conditioned medium or Ménéz's B2 (Stojkovic *et al.*,

1995a). Therefore, GLCM was used to produce trophoblastic vesicles for the present study. However, after day 15 these trophoblastic vesicles derived *in vitro* could be maintained alive in serum-free M-199, providing the basis for the analysis of embryotrophic effects of trophoblast secretory products. Some trophoblastic vesicles were cultured in serum-free M-199 for 3 weeks without any morphological sign of degeneration (data not shown). Hernandez-Ledezma *et al.* (1992) found that long-term survival and secretion of IFN τ of bovine embryos produced in M-199 was dependent on transient exposure to a uterine environment.

The positive effect of TVCM on early embryonic cleavage and development to the blastocyst stage observed in the present study is in line with findings by Heyman *et al.* (1987), who evaluated media conditioned by trophoblastic vesicles derived *ex vivo* for their potential to support *in vitro* development of bovine embryos derived *ex vivo*. These authors found conditioned medium to be equally effective as coculture with trophoblastic cells. However, this conclusion was drawn from a relatively small number of cultured embryos which were not monitored up to the blastocyst stage. Trophoblastic vesicles derived from *ex vivo* embryos release one or several unknown compounds normally present *in vivo* (Wildeman *et al.*, 1991), promoting cleavage of early bovine embryos *in vitro* (Camous *et al.*, 1984). After ultrafiltration of media, the main

embryotrophic activity was in the low molecular mass fraction both in our study and in the study on trophoblastic secretions *ex vivo* by Heyman *et al.* (1987). By contrast, analysis of bovine oviduct epithelial cell-conditioned medium (BOECM) revealed that a low molecular mass fraction (< 10 kDa) maintained development to the 5–8-cell stage but did not support development to the blastocyst stage (Vansteenbrugge *et al.*, 1996). The latter was stimulated by high molecular mass (> 10 kDa) factors (Mermillod *et al.*, 1993). Similarly, the embryotrophic activity of human tubal fluid conditioned with bovine granulosa cells was partially or completely removed by 30 kDa or 10 kDa ultrafiltration, respectively (Maeda *et al.*, 1996). The proportion of inseminated oocytes developing to the 5–8-cell stage in serum-free TVCM is in the same range as in other studies involving embryo culture in serum-free BOECM (Mermillod *et al.*, 1993) or human tubal fluid conditioned by bovine granulosa cells or Vero cells (Maeda *et al.*, 1996). The rate of development to the blastocyst was significantly higher in TVCM than in M-199 in all three culture conditions evaluated. The fact that TVCM increased blastocyst rate in the presence of serum and cumulus cells suggests that trophoblastic cells derived *in vitro* secrete important factors complementing those provided by cumulus cells or those in the serum.

TVCM contained 35 µg total protein ml⁻¹ which is in the same range as protein concentrations of serum-free media conditioned with bovine oviduct epithelial cell monolayers (Mermillod *et al.*, 1992; Vansteenbrugge *et al.*, 1996) or with bovine granulosa cells (Maeda *et al.*, 1996). The blastocyst rate obtained with serum-free TVCM averaged 21%. Blastocyst rates obtained in serum-free BOECM varied between 27% (Mermillod *et al.*, 1993) and 19% (Vansteenbrugge *et al.*, 1996). In contrast to our findings with TVCM, no additive effects between serum and BOECM were observed by Vansteenbrugge *et al.* (1996).

In addition to rates of development to the 5–8-cell and blastocyst stages, the effects of TVCM on the number of cells in blastocysts were studied. The number of ICM cells was greatest in blastocysts produced in the cumulus cell coculture system, followed by the cell-free serum containing media and the serum-free media. A positive effect of TVCM on the number of ICM cells was only significant in the serum-free system. By contrast, there was no or even a negative effect (in the presence of serum) of TVCM on the number of TE cells, suggesting a specific mitogenic effect of TVCM on ICM cells. Iwasaki *et al.* (1990) reported the number of ICM cells as between 8 and 16 for bovine blastocysts (day 8–12) produced *in vitro* in a cumulus cell coculture system. In the present study, blastocysts cultured for 8 days in serum-free TVCM had 15–32 ICM cells. In other studies on serum-free conditioned media for bovine embryo culture, only the total number of cells in blastocysts have been reported. Mean total numbers of cells in day 9 blastocysts produced in human tubal fluid conditioned with bovine granulosa cells or Vero cells were 97 and 94, respectively (Maeda *et al.*, 1996). The average total number of cells in day 8 blastocysts cultured in TVCM was 69 in the absence and 79 in the presence of serum. Mermillod *et al.* (1993) reported that day 7 bovine blastocysts after culture in serum-free or serum containing BOECM had the same total number of cells (110).

In summary, the present study shows that TVCM contains potent embryotrophic factors which act complementary to those secreted by cumulus cells and those supplemented with serum, and result in reliably high blastocyst rates of almost 40%. Since contamination of TVCM with serum proteins can be avoided, this medium may be a reasonable source for the purification of specific embryotrophic factors.

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References

- Camous S, Heyman Y, Meziou W and Ménézo Y (1984) Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles *Journal of Reproduction and Fertility* **72** 479–485
- Flint APF, Henville A and Christie WB (1979) Presence of placental lactogen in bovine conceptuses before attachment *Journal of Reproduction and Fertility* **56** 305–308
- Handyside AH and Hunter S (1984) A rapid procedure for visualising the inner cell mass and trophectoderm nuclei of mouse blastocysts *in situ* using polynucleotide-specific fluorochromes *Journal of Experimental Zoology* **231** 429–434
- Hernandez-Ledezma JJ, Sikes JD, Murphy CN, Watson AJ, Schultz GA and Roberts RM (1992) Expression of bovine trophoblast interferon in conceptuses derived by *in vitro* techniques *Biology of Reproduction* **47** 374–380
- Heyman Y, Ménézo Y, Chesné P, Camous S and Garnier V (1987) *In vitro* cleavage of bovine and ovine early embryos: improved development using coculture with trophoblastic vesicles *Theriogenology* **1** 59–68
- Iwasaki S, Yoshida N, Ushijima H, Watanabe S and Nakahara T (1990) Morphology and proportion of inner cell mass of bovine blastocysts fertilized *in vitro* and *in vivo*. *Journal of Reproduction and Fertility* **90** 279–284
- Liu KH, Dore JJE, Roberts MP, Krishnan R, Hopkins FM and Godkin JD (1993) Expression and cellular localisation of retinol-binding protein messenger ribonucleic acid in bovine blastocysts and extraembryonic membranes *Biology of Reproduction* **49** 393–400
- Maeda J, Kotsuji F, Negami A, Kamitani N and Tominaga T (1996) *In vitro* development of bovine embryos in conditioned media from bovine granulosa cells and Vero cells cultured in exogenous protein- and amino acid-free chemically defined human tubal fluid medium *Biology of Reproduction* **54** 930–936
- Mermillod P, Mourmeaux J, Wils C, Massip A and Dessy F (1992) Protein-free oviduct conditioned medium for complete bovine embryo development *Veterinary Record* **130** 13 (Abstract)
- Mermillod P, Vansteenbrugge A, Wils C, Mourmeaux J, Massip A and Dessy F (1993) Characterization of the embryotrophic activity of exogenous protein-free oviduct-conditioned medium used in culture of cattle embryos *Biology of Reproduction* **49** 582–587
- Morgan G, Wooding FBP, Beckers J and Friesen HG (1989) An immunological cryo-ultrastructural study of a sequential appearance of proteins in placental binucleate cells in early pregnancy in the cow *Journal of Reproduction and Fertility* **86** 745–752
- Papaioannou VE and Ebert KM (1988) The preimplantation pig embryo: cell number and allocation to trophectoderm and inner cell mass of the blastocyst *in vivo* and *in vitro*. *Development* **102** 793–803
- Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH and First NL (1986) Bovine *in vitro* fertilization with frozen-thawed semen *Theriogenology* **25** 591–600
- Roberts RM, Cross JC and Leaman DW (1992) Interferons as hormones of pregnancy *Endocrine Reviews* **13** 432–452
- Solter D and Knowles BB (1975) Immunology of mouse blastocyst *Proceedings of National Academy of Science USA* **72** 5099–5102
- Stojkovic M, Wolf E, Büttner M, Berg U, Charpigny G, Schmitt A and Brem G (1995a) Secretion of biologically active interferon tau by *in vitro*-derived bovine trophoblastic tissue *Biology of Reproduction* **53** 1500–1507
- Stojkovic M, Willemsen D, Berg U, Brem G and Wolf E (1995b) Effect of coculture with *in vitro* derived trophoblastic vesicles on the development of

- in vitro* produced bovine embryos *Biology of Reproduction* **52** Supplement **1** 128 (Abstract)
- Stojkovic M, Büttner M, Zakhartchenko V, Brem G and Wolf E A reliable procedure for differential staining of *in vitro* produced bovine blastocysts: comparison of tissue culture medium 199 and Ménézo's B2 medium *Animal Reproduction Science* (in press)
- Vansteenbrugge A, Van Langendonck A, Donnay I, Massip A and Dessy F (1996) Effect of high molecular weight factors present in bovine oviduct-conditioned medium on *in vitro* bovine embryo development *Theriogenology* **46** 631–641
- Wildeman AG, Sanderson K, Scodras JM and Betteridge KJ (1991) Developmental changes in protein synthesis by day 14–17 bovine conceptuses, trophoblastic vesicles, and a derived cell line *Journal of Reproduction and Fertility Supplement* **43** 50–51
- Xie S, Low BG, Nagel RJ, Beckers J and Roberts RM (1994) A novel glycoprotein of the aspartic proteinase gene family expressed in bovine placental trophoctoderm *Biology of Reproduction* **51** 1145–1153
- Zoli AP, Guilbault PD, Ortiz WB and Beckers J-F (1992) Radioimmunoassay of bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis *Biology of Reproduction* **46** 83–92