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A reliable procedure for differential staining of in vitro produced bovine blastocysts: comparison of tissue culture medium 199 and Ménézo's B2 medium

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Abstract

A reliable double-dye technique has been established for counting the number of inner cell mass and trophectoderm cells of in vitro produced bovine blastocysts. The latter were first incubated in a 1:2 dilution of a rabbit antiserum raised against a mixture of recombinant bovine interferon tau and serum containing medium conditioned by in vitro produced trophoblastic vesicles for 45 min at 39°C. Subsequently, the blastocysts were incubated in a 5% (v/v) solution of guinea pig complement in phosphate-buffered saline containing 50 μg/ml propidium iodide for 45 min at 39°C. Then the blastocysts were transferred to ice-cold absolute ethanol containing 25 μg/ml bisbenzimide and evaluated under a fluorescence microscope. Since trophectoderm cells were permeabilised by antibody-mediated complement lysis, they were stained by propidium iodide (red or pink). Bisbenzimide can enter lysed and non-lysed cells and therefore stained also inner cell mass cells (blue) which had been protected from complement lysis by trophectoderm cells. This modified procedure proved to be very reliable for differential cell staining of bovine blastocysts produced under various culture conditions. A comparison of blastocysts produced in Ménézo's B2 vs. TCM 199 media (both supplemented with 10% serum from cows at oestrus) revealed significant ($P < 0.01$) differences in total cell numbers (119 ± 24 vs. 84 ± 10; mean $\pm$

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SD) and in the numbers of trophectoderm cells (79 ± 19 vs. 57 ± 8) and inner cell mass cells (40 ± 7 vs. 26 ± 5) between the two culture systems. The modified staining procedure presented here is a valuable tool for evaluating the quality of in vitro produced bovine blastocysts and for improving of culture conditions.

Keywords: Cattle; Embryology; Inner cell mass; Trophectoderm; Interferon

1. Introduction

The bovine blastocyst consists of two morphologically distinct cell types: 1) inner cell mass (ICM) cells and 2) an outer monolayer of trophectoderm (TE) cells. The ICM will differentiate into all tissues of the developing fetus. The TE is responsible for the vectorial transport of fluid during blastocyst formation, for maternal recognition of pregnancy, attachment and invasion of the uterine endometrium during implantation. After implantation, TE cells give rise to the placenta and extra-embryonic membranes (Gardner et al., 1973). The dominant secretory product of pre-implantation bovine embryos in vivo (Roberts et al., 1992) and in vitro (Stojkovic et al., 1995) is interferon tau (IFNτ). The latter is secreted specifically by TE cells and represents the key molecule for maternal pregnancy recognition, preventing regression of the corpus luteum (Bazer et al., 1994).

The number of ICM and TE cells may be a valid indicator of viability and quality of mammalian blastocysts (Papaioannou and Ebert, 1988). Consequently, methods for differential cell staining have been applied for embryos from different species, including mouse (Solter and Knowles, 1975; Handyside and Hunter, 1984), rabbit (Giles and Foote, 1995), pig (Papaioannou and Ebert, 1988; Boerjan and te Kronnie, 1993; Tao et al., 1995; Rivera et al., 1996), human (Hardy et al., 1989) and bovine (Iwasaki et al., 1990; Iwasaki et al., 1994; Narula et al., 1996; van Soom et al., 1996). The basic principle of the double-dye technique involves lysis of TE cells by an antibody-mediated complement reaction and subsequent staining of TE nuclei and ICM cells with non-penetrating (propidium iodide = PI) and penetrating (bisbenzimide = BB) polynucleotide-specific fluorochromes (Papaioannou and Ebert, 1988). Other studies involving differential cell counts of bovine blastocysts used rabbit antiserum raised against bovine spleen cells (Iwasaki et al., 1990; Iwasaki et al., 1994; Narula et al., 1996). In our hands, this procedure was not reliable for in vitro produced embryos. Therefore, we used a rabbit antiserum raised against a mixture of recombinant bovine IFNτ and serum containing medium conditioned by in vitro produced trophoblastic vesicles (Stojkovic et al., 1995). This modified technique was highly reproducible and clearly showed differences between two routinely used media for in vitro embryo culture, Ménézo's B2 and TCM 199.

2. Materials and methods

All chemicals were obtained from Sigma (St. Louis, MO), unless stated otherwise.

2.1. Development of a rabbit antiserum for trophoblast lysis

A rabbit antiserum for trophoblast lysis was developed by conventional methods (Solter and Knowles, 1975). Briefly, 1000 antiviral units of a single recombinant form of bovine IFNτ (Klemann et al., 1990) were dissolved in 0.5 ml TE cell conditioned Dulbecco's MEM medium (Biochrom, Berlin, Germany) containing 10% foetal calf serum (Biochrom, Berlin, Germany) and 10 000 antiviral units of IFNτ secreted by in vitro produced trophoblastic vesicles (Stojkovic et al., 1995). For immunisation, the antigen was mixed (1:1, by volume) with Freund's complete adjuvant and injected subcutaneously into four male Siberian rabbits. Two booster injections were made at four week intervals. The first blood sample was taken one week after the second antigen injection. Complete serum was collected from each animal two weeks after the last booster injection, heat inactivated (56°C, 30 min) and frozen at -20°C until using.

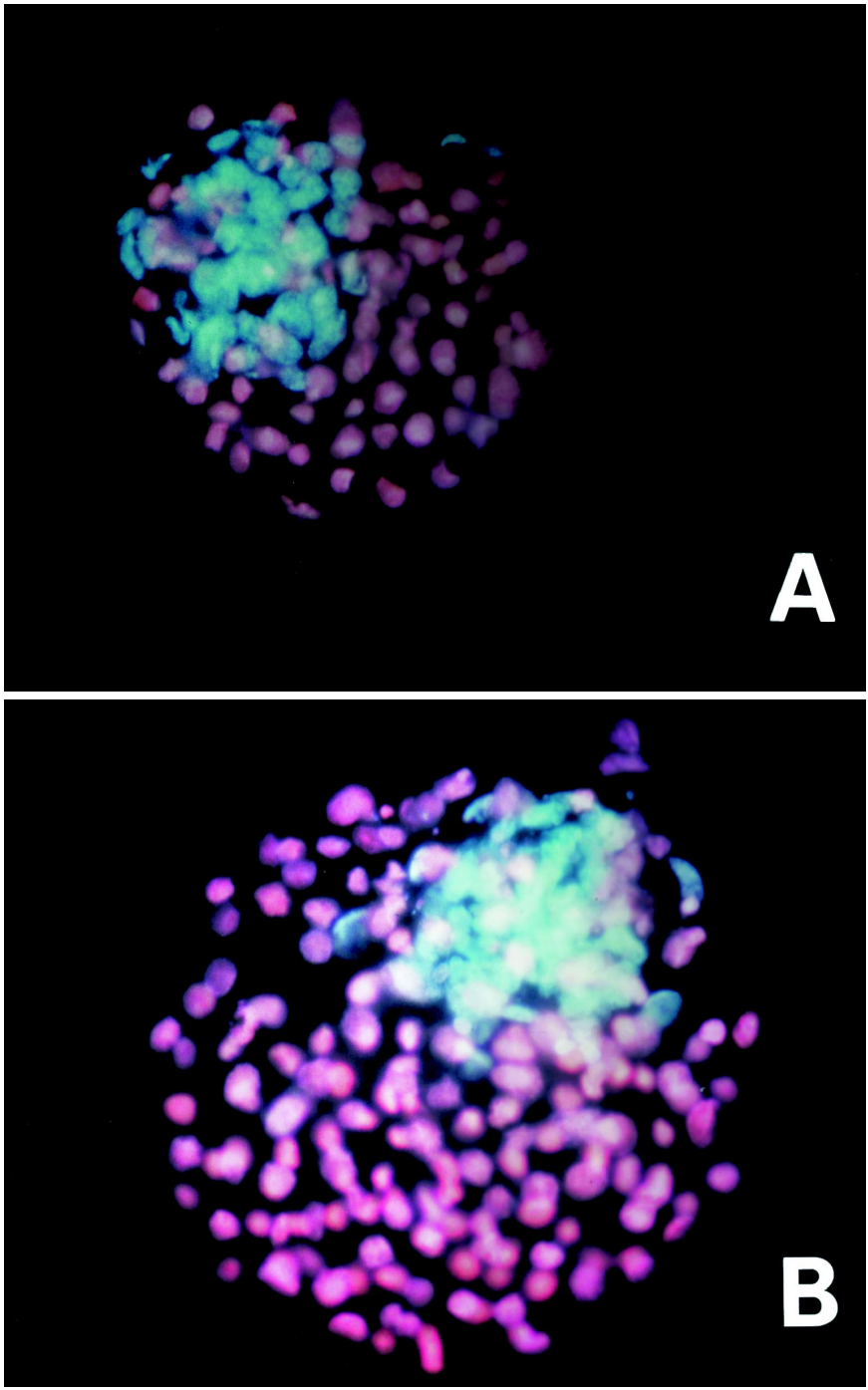
2.2. In vitro production of bovine blastocysts

Bovine blastocysts were produced in vitro as previously described (Stojkovic et al., 1995). Briefly, ovaries were recovered from slaughtered cows at the local abattoir. Oocytes were collected by aspiration of 2–8 mm follicles. Only oocytes with a complete dense cumulus and a dark cytoplasm were selected for in vitro maturation (IVM). The medium used for IVM was TCM 199 (Biochrom, Berlin, Germany) supplemented with 4.43 mM Hepes, 33.9 mM sodium bicarbonate, 2 mM sodium pyruvate, 2.92 mM calcium lactate, 10 μg/ml porcine follicle stimulating hormone (FSH-p; Schering Pharmaceuticals, Kenilworth, NJ), 60 μg/ml gentamicin and 10% (v/v) heat-inactivated serum from cows at oestrus (ECS). Groups of 30–40 oocytes were matured for 24 h in 400 μl maturation medium at 39°C in an atmosphere of 5% CO_2 in air and maximum humidity. After maturation, oocytes were transferred into TALP medium containing 6 mg/ml bovine serum albumin, 10 μg/ml heparin, and frozen/thawed semen (10^6 spermatozoa/ml). Motile spermatozoa were obtained by swim-up procedure

Table 1
Effect of Ménézo's B2 and TCM 199 medium on the developmental rate of in vitro matured and in vitro fertilized bovine oocytes

Culture medium	Ménézo's B2	TCM 199
No. of replicates	5	5
No. of oocytes	690	673
No. (% ± SEM) of 5–8 cells (66 hpi)	566 (82 ± 1.4)	527 (78 ± 1.4)
No. (% ± SEM) of blastocysts (162 hpi)	268 (39 ± 0.9)[a]	205 (31 ± 1.4)[b]
No. (% ± SEM) of blastocysts (186 hpi)	290 (42 ± 2.2)[a]	218 (32 ± 2.9)[b]
Blastocyst	34 (5 ± 0.7)	43 (6 ± 0.7)
Expanded	173 (25 ± 1.0)[a]	128 (19 ± 1.1)[b]
Hatched	83 (12 ± 1.2)[a]	47 (7 ± 1.1)[b]

Proportions marked by different superscripts within rows are significantly different (χ^2 test; $P < 0.01$).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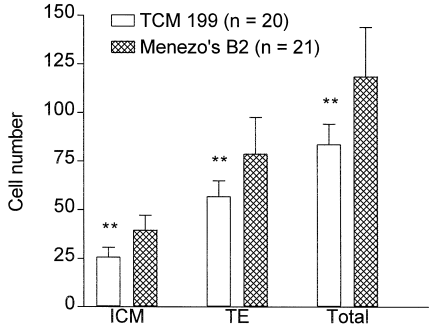

Fig. 2. Numbers of inner cell mass (ICM) and trophectoderm cells (TE) and total cell numbers of blastocysts cultured in TCM 199 and Ménézo's B2 media. Blastocysts were analysed on Day 8 (fertilization = Day 0); n = number of embryos analysed. Cell numbers were compared using Mann–Whitney U-tests: $**P < 0.01$.

(Parrish et al., 1986). After 18 h, cumulus cells were removed from presumptive zygotes by vortexing them for 90 s in 0.8 ml culture media: Ménézo's B2 (bioMérieux, Marcy-l'Etoile, France) or TCM 199, both supplemented with 10% ECS. In five replicates, randomly divided embryos were washed in culture media and groups of 30–35 embryos transferred in 400 μl of culture medium containing 5×10^5 vortexed cumulus cells under paraffin oil (Merck, Darmstadt, Germany) at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2. The cleavage rate (5–8 cell stage) was evaluated 66 h post insemination (hpi), and the number of blastocysts 162 and 186 hpi. On Day 8 (fertilization = Day 0), expanded and hatched blastocysts were used for immunostaining.

2.3. Double-dye technique for differential ICM and TE cell counts of bovine embryos

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) in PBS for 3–5 min to dissolve the zona pellucida. Zona-free and hatched blastocysts were incubated in a 1:2 dilution of rabbit antiserum in PBS for 45 min at 39°C in a humidified atmosphere of 5% CO_2 in air. Subsequently, embryos were washed 5 times in PBS (39°C) and then incubated in PBS supplemented with 5% (v/v) guinea pig complement (ICN Immunobiologicals, Costa Mesa, CA) and 50 μg/ml propidium iodide (PI) for 45 min at 39°C in a humidified atmosphere of 5% CO_2. This step resulted in selective antibody-mediated complement lysis of trophectoderm cells (Solter and Knowles, 1975) and staining of trophectoderm cell nuclei (red). After this step, blastocysts were washed again in PBS and then placed in cold absolute

Fig. 1. Differentially stained expanded (A) and hatched (B) bovine blastocysts cultured in TCM 199 (×400). Nuclei of inner cell mass cells show blue fluorescence, whereas nuclei of trophectoderm cells are stained pink.

ethanol (Merck, Darmstadt, Germany) containing 25 μg/ml bisbenzimide (BB; Riedel-Haen, Seelze, Hannover, Germany) for 30 min at 4°C. This resulted in the staining of all cell nuclei (intact inner cell mass and lysed trophectoderm) and the fixation of the embryo. Finally, embryos were washed in absolute ethanol, then mounted in undiluted glycerol (Merck, Darmstadt, Germany) and squashed on a glass slide. The stained embryos were observed and ICM and TE cells were counted directly using a fluorescent microscope (Axiovert 135, Zeiss, Jena, Germany) with an HBO lamp under transmitted illumination and an ultraviolet excitation filter of 365 nm and a barrier filter of 420 nm. Bisbenzimide-stained vital ICM nuclei appeared blue, and non-vital TE nuclei, which were stained with both bisbenzimide and propidium iodide, gave red or pink fluorescence. Photomicrographs were taken with a Contax 139 Quartz automatic camera system using Fujichrome colour daylight film (400 ASA).

3. Results

3.1. Cleavage and blastocyst rates of bovine embryos produced in Ménézo's B2 and TCM 199 media

The results are given in Table 1. Ménézo's B2 was not superior to TCM 199 medium with respect to early in vitro development (5–8 cell stage) when used in a co-culture system with cumulus cells. By contrast, blastocyst rates were significantly ($P < 0.01$) different between the two culture media: 39% and 42% vs. 31% and 32% of oocytes developed to the blastocyst stage 162 hpi and 186 hpi in Ménézo's B2 and TCM 199, respectively.

3.2. Differential staining and cell counts of in vitro produced bovine blastocysts

From 46 embryos stained for a comparison of two standard culture media, 40 embryos could be clearly evaluated (86.9%). Of these embryos, 11 hatched and 9 expanded blastocysts (from 22) recovered in TCM 199, and 12 hatched and 9 expanded blastocysts (from 24) recovered in Ménézo's B2 were evaluated for number of ICM and TE cells. Representative pictures of a double stained expanded (A) and hatched (B) blastocysts are shown in Fig. 1.

Total cell numbers (118.6 ± 25.3 vs. 83.8 ± 10.3), numbers of ICM (39.7 ± 7.4 vs. 25.9 ± 4.7) and TE (78.9 ± 18.6 vs. 56.9 ± 7.9) cells of analysed blastocysts cultured in Ménézo's B2 were significantly ($P < 0.01$) greater than of those cultured in TCM 199 (Fig. 2).

4. Discussion

The survival of bovine embryos in vivo depends on the number and viability of ICM cells and on the ability of TE cells to secrete IFNτ which plays an important role in maternal recognition of pregnancy by its antiluteolytic effect (Roberts et al., 1992).

Since cell number is a valid indicator of embryo quality (Papaioannou and Ebert, 1988), differential cell staining of blastocysts is an important technique for embryo biotechnology, e.g. for evaluating culture conditions. The number of cells in both ICM and TE can be determined for individual blastocysts and regional interactions can be analysed (Handyside and Hunter, 1984). Differential staining of ICM and TE cells depends on the assumption that in a zona-free embryo the outer cells form a seal which prevents diffusion of antibodies to inner cells, and on complement-mediated lysis of these outer cells exposed to specific antiserum. The barrier for non-selective uptake of macromolecules probably consists of the TE cells themselves and the tight junction between them (Solter and Knowles, 1975).

We have developed a protocol for differential cell staining of bovine blastocysts which uses antiserum to a mixture of foetal calf serum and secretory products of TE cells which proved to be very reliable. It is unlikely that the efficient antibody-mediated complement lysis was due to antibodies against IFNτ since an antiserum raised against pure recombinant bovine IFNτ (kindly provided by Dr. R.M. Roberts) was not effective in this procedure (data not shown). Other authors described that in some cases the discrimination between ICM and TE cells was difficult (Narula et al., 1996) and that in frozen-thawed embryos some ICM cells appeared pink (Iwasaki et al., 1994). The authors of the latter study concluded that these pink cells were dead cells among the blue vital nuclei of the ICM. We found that blastocysts with some degenerated TE cells (dark cells) appeared blue after double-staining. Narula et al. (1996) reported that slow-developing buffalo blastocysts have a tendency to disintegrate during incubation in the complement. In our study, we did not observe any differences between expanded (slow-developing) and hatched (fast-developing) blastocysts. In contrast to other investigators (Iwasaki et al., 1990; Iwasaki et al., 1994; Narula et al., 1996), we used PBS without serum or protein supplementation for the dilution of antiserum and complement/PI and as washing medium as well. Foetal calf serum which was used in other studies may contain factors which interact with antibody binding, complement lysis or staining of TE and ICM cell nuclei by fluorochromes. Blastocysts mounted in glycerol could not be stored successfully for more than three days because of gradual dissociation of the fluorochromes and loss of specific labelling. This is consistent with findings from Handyside and Hunter (1984).

We employed our modified double staining technique to compare two routinely used media for bovine embryo culture, Ménézo's B2 and TCM 199 (Xu et al., 1992; Farin et al., 1995; van Soom et al., 1996). Ménézo's B2 was not superior to TCM 199 medium with respect to early in vitro development (5–8 cell stage) when used in a co-culture system with cumulus cells. This is consistent with the results obtained by Xu et al. (1992) and Merton et al. (1995) but contrasts findings by van Soom et al. (1996). Based on a coculture system with bovine oviduct cells, the latter study revealed a higher proportion of more 8-cell stages on Day 3 in Ménézo's B2 than in TCM 199.

Blastocyst rates 186 hpi were significantly higher in Ménézo's B2 than in TCM 199. This is in agreement with results obtained later (Merton et al., 1995; van Soom et al., 1996). Both, Ménézo's B2 and TCM 199 are complex media, but there are some differences in composition of proteins, mineral salts, amino acids, lipids, metabolic by-products and vitamins (van Soom et al., 1996). In contrast to TCM 199, Ménézo's

B2 contains 10 g/L BSA. It is suggested that BSA could be serving as a 'covered' energy source. This suggestion is supported by the observation that BSA can be taken up by endocytosis and partly degraded by pre-implantation embryos (Barnett and Bavister, 1995).

Total cell numbers and number of TE cells of expanded and hatched blastocysts cultured in Ménézo's B2 were significantly greater than of those cultured in TCM 199. This is in line with findings of a recently published study by van Soom et al. (1996). This group reported that expanded and hatched blastocysts produced in Ménézo's B2 possess more TE cells than embryos produced in TCM 199. By contrast, the numbers of ICM cells were not different between embryos produced in the two media. However, only one blastocyst produced in TCM 199 and five blastocysts produced in Ménézo's B2 were evaluated. Based on the larger number of embryos, our study clearly showed that Ménézo's B2 medium has a positive effect on the number of both TE and ICM cells on in vitro produced bovine blastocysts.

Iwasaki et al. (1990) reported ICM cell numbers between 11 and 16 for in vitro produced expanded and hatched bovine blastocysts (Day 8–12), cultured in TCM 199 and cumulus cell co-culture. In the same study, after 2–3 days in vitro culture of in vivo Day 7 flushed embryos, expanded and hatched blastocysts possess 23 ± 7 and 31 ± 12 ICM cells, respectively. In an other study (Du et al., 1996) was shown that Day 9 flushed hatched blastocysts contain 118 ± 8 ICM cells.

In conclusion, our modified double-staining procedure revealed clear differences in cell numbers of blastocysts cultured in Ménézo's B2 vs. TCM 199. This technique may be a valuable tool for improving conditions of in vitro embryo culture.

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