Conditioned medium increases the polyploid cell composition of bovine somatic cell nuclear-transferred blastocysts

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Abstract

The effects of bovine cumulus cell-conditioned medium on cloned bovine embryonic development and subsequent chromosome complement were examined using an air-dry procedure. Conditioned media were prepared using CR1aa supplemented with either fetal bovine serum (FBS) or bovine serum albumin (BSA). Nuclear-transferred embryos were reconstructed with nuclei from cumulus cells. Similar cleavage, morula, and blastocyst development was observed in conditioned media groups compared with the co-culture group. No differences ($P > 0.05$) were observed in the composition of blastocyst chromosomes after co-culture in different media, either with or without starvation of donor cells. The overall diploid blastocyst rate ranged from 75% to 84%. Chromosomal complement of blastocysts, however, was very different between conditioned medium and co-culture treatments. Overall incidence of chromosomal anomalies was 40% in conditioned medium, which was significantly higher ($P < 0.001$) than the co-culture group (20%). Moreover, a higher incidence ($P < 0.05$) of chromosomally abnormal blastocysts (41.5%) was observed after culture with FBS-containing conditioned medium than those cultured in BSA-containing conditioned medium (31.4%). No diploid improvement was observed after exchange of the culture system from conditioned medium to co-culture, or from co-culture to conditioned medium after the first 72 h of culture. The results of this study also indicated that the overall cell number was much lower ($P < 0.01$) in blastocysts with chromosomal abnormalities than those with a normal diploid state. We have concluded that medium conditioned with bovine cumulus cells increases the incidence of chromosomal anomalies in nuclear reconstructed embryos.

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Introduction

Successful somatic cell nuclear transfer (NT) has now been achieved in several mammalian species as reported by the birth of offspring. The overall efficiency of the technique, however, remains low. In each animal species so far studied, less than 10% of transferred embryos typically survive to term (Hill 2002). A high frequency of early post-implantation developmental arrest and abortion exists, especially in cattle. Studies in cattle and sheep have shown the most significant period for fetal loss to be at the time of placental attachment, which occurs between the first and second month of gestation (Hill 2002). The exact mechanism(s) of these phenomena is still unclear. The NT procedures, i.e. removal of oocyte nuclear material, transfer of a differentiated cell into an enucleated cytoplasm, fusion with electric pulse, chemical or electric pulse activation, and culture conditions, can all lead to potential cell cycle blockages. All of these artificial manipulations can potentially influence the chromosomal composition of the resulting embryos. Approximately half

of all human spontaneous abortions in the first and second trimesters are chromosomally abnormal (Burgoyne *et al.* 1991). Only a few reports have been published on the chromosome analysis of bovine NT embryos (Mohamed & Takahashi 2000, Arat *et al.* 2002, Slimane-Bureau & King 2002, Booth *et al.* 2003, Slimane-Bureau *et al.* 2003).

Although bovine embryos can be cultured *in vitro* in simple media under defined conditions (Keskintepe *et al.* 1995, Takahashi *et al.* 1996), the addition of growth factors facilitates embryo development (Paria & Dey 1990, O'Neill 1997), which suggests that paracrine and/or autocrine pathways exist between helper cells and embryos. To mimic the *in vivo* environment, co-culture systems are widely used in the culture of *in vivo* or *in vitro* fertilized embryos (Van Langendonck *et al.* 1996, Funston *et al.* 1997, Vanroose *et al.* 2001). Various studies have reported that co-culture improves the morphological development and implantation rate of exposed embryos. Numerous growth factors, receptors, and binding proteins are secreted into the medium by helper cells. Embryos have also been reported to secrete substances into their

surrounding environment (Van Langendonck *et al.* 1996, Izquierdo *et al.* 1999). Cell-free conditioned medium (CM) has been used in several laboratories in the culture of *in vivo* and *in vitro* fertilized embryos (Baranao *et al.* 1997, Funston *et al.* 1997, Ishiwata *et al.* 2000, Galli *et al.* 2003). Studies have shown that CM has similar effects on embryo development to those produced in various co-culture systems. It is thought that the CM system may eliminate partially, or completely, some of the *in vitro* culture artifacts, e.g. developmental blockage and fragmentation.

Currently, there are no reports on the chromosome complement of NT blastocysts derived from CM culture. This study was designed to determine: (1) the effects of medium conditioned by bovine cumulus cells on the subsequent development of NT bovine embryos and (2) the chromosome complements of NT blastocysts after CM culture.

Materials and Methods

Chemicals were purchased from Sigma Chemical Co. unless otherwise indicated.

Oocyte maturation in vitro (IVM)

Maturation of bovine oocytes was carried out according to routine procedures (Li *et al.* 2003). Briefly, bovine cumulus oocyte complexes (COCs) were aspirated from 3–8 mm diameter follicles from ovaries obtained from a local abattoir. Only COCs with a compact cumulus complex and a homogenous ooplasm were selected. The COCs were matured in TCM 199 with Earle's salts, L-glutamine and sodium bicarbonate (Gibco Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA, 25 µg/ml gentamicin, 0.01 U/ml follicle-stimulating hormone (NIH-FSH-S17), 0.01 U/ml luteinizing hormone (USDA-bLH-6) and 1 µM penicillamine, hypotaurine and epinephrine (PHE) in four-well plates with 0.5 ml medium and 30–50 oocytes per well under 39 °C in humidified 5% CO₂ in air for 18–19 h.

Donor cell culture and preparation

Bovine cumulus cells were used as nuclear donor cells. A primary cumulus cell line was established from six oocytes collected 18–20 h after the start of maturation. Cumulus cells were separated with 0.1% hyaluronidase (type-1), washed several times in Dulbecco's modified Eagles' medium (DMEM)/F12 (1:1) (Gibco Inc.), and cultured in DMEM/F12 supplemented with 10% FBS in 25 cm flasks at 37 °C under 5% CO₂ in air. The cells were passed at 80% or more confluence. They were starved by culture in 0.5% FBS for 3–5 days. The non-starved cells were cultured in normal medium with 10% serum, and the 80% confluent cells were used as donors. Immediately before NT, cells were dissociated by trypsinization with 0.25% trypsin with EDTA solution (HyClone). Small sized (10–12 µm) cells with a smooth round shape from both

starved and non-starved groups were used as nuclear donor cells.

Preparation of CM

CM was prepared from fresh CR1aa exposed to cumulus cell cultures for 24 h. Twenty-four hours after passaging in DMEM/F12 + 10% FBS (cells in one flask were seeded into four 25 cm² flasks, once those cells reached 70%–80% confluence in 24 h), the flasks were washed twice with CR1aa, and then 3 ml fresh CR1aa supplemented with 3% FBS (serum-containing CM; SCM) or 0.3% fatty acid free bovine serum albumin (BSA) (BSA-containing CM; BCM) was added. The SCM and BCM were collected 24 h after seeding and subsequently filtered (0.2 µm) to remove cellular debris and stored at 4 °C for less than 2 weeks.

Enucleation and NT

After maturation, the cumulus cells were removed by vortexing COCs in TL-Hepes (Tyrode lactate Hepes buffered) medium containing 100 IU/ml hyaluronidase. Removal of chromosomes was carried out with the assistance of colcemid (Gibco) as described by Yin *et al.* (2002). Briefly, oocytes with first polar bodies were cultured into TCM 199 supplemented with 0.2 µg/ml colcemid for 2 h. The eggs with membrane protrusions were transferred into TL-Hepes medium containing 7.5 µg/ml cytochalasin B and 0.1 µg/ml colcemid and then the protrusion and the first polar body were removed by a beveled pipette. Single cells were individually transferred to the perivitelline space of the recipient cytoplasts.

Fusion and activation

Fusion was performed using one direct current pulse of 1.2 kV/cm for 25 µs by an Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA) in 0.27 M mannitol, 0.1 mM CaCl₂, 0.1 mM MgCl₂, and 0.05% BSA. Fused eggs were activated 24–25 h after the start of IVM with 5 µM ionomycin for 5 min, followed by treatment with 10 µg/ml cycloheximide in CR1aa plus 3% FBS for 5 h at 38.5 °C in 5% CO₂ in air.

In vitro culture of NT embryos

After activation, NT embryos were cultured in 30 µl droplets of chemically defined medium (CDM) (Olson & Seidel 2000, Li *et al.* 2003), or CR1aa supplemented with 3% FBS and co-cultured with monolayer bovine cumulus cells in experiment 1, or cultured in SCM or BCM in experiments 2 and 3 overlaid with mineral oil at 39 °C in 5% CO₂ in air for 7 days. Cleavage, morula and blastocyst stages were evaluated at 48 h, day 5 and day 7 after activation (day 0) respectively.

Chromosomal analysis and cell number count

Day 7 blastocysts were prepared and examined for their cytogenetic composition and the number of nuclei contained within each embryo. Blastocysts were incubated in 0.05 µg/ml colcemid in culture medium for 5 h.

The blastocysts were then treated with 1% trisodium citrate for 10–15 min, and transferred individually onto a clean glass slide and fixed (Li *et al.* 1998, 2003). Slides were stained with 1% Giemsa for 10 min. Chromosome spreads were examined at 1000 $\times$ under oil, and the chromosome composition was determined for each blastocyst. Images were captured by digital camera with PIXERA Viewfinder Program (Pixera Corporation, Los Gatos, CA, USA) under a Zeiss microscope. The categories for the chromosome composition were classified as follows: diploid, polyploid (tetraploid), mixoploid (including 1n/2n, 2n/3n, 2n/4n, 2n/3n/4n), and aneuploid. Only those embryos with over four chromosome spreads were scored and analyzed. Embryos that did not show an interpretable metaphase spread because of gross overspreading or clumped chromosomes were not classified.

Experimental design

Three experiments were designed. Experiment 1 examined the effect of different media on blastocyst development of NT embryos and their chromosomal compositions. Fused NT embryos from starved or non-starved donor cells were randomly cultured in CDM or CR1aa medium with co-culture. On day 7 of culture, blastocysts were prepared for chromosomal analysis. Because no differences in blastocyst development and chromosomal composition between starved and non-starved donor cells were observed in experiment 1, only non-starved cells were used in experiments 2 and 3.

Experiment 2 evaluated the effects of BCM and SCM on development and chromosomal composition of NT blastocysts. Fused NT embryos were randomly cultured in SCM and BCM, and CR1aa + 3% FBS with co-culture as control.

Experiment 3: because of the effect of CM on blastocyst chromosomal composition in experiment 2, this experiment was designed to test whether changes of the culture system from co-culture to SCM or from SCM to co-culture after the first 72 h of culture would improve blastocyst

diploid complements. Fused NT embryos were co-cultured in CR1aa + 3% FBS for 72 h, then transferred into fresh SCM for further culture (from co-culture to SCM; CO–SCM), or embryos were cultured in SCM for the first 72 h, then transferred into the co-culture system up to day 7 (from SCM to co-culture; SCM–CO). Embryos that were cultured during the entire culture period in co-culture or in SCM for 7 days were used as the controls.

Statistical analysis

Differences in blastocyst cell numbers between groups were analyzed by one-way ANOVA. Differences in rates of blastocyst development and chromosome composition between experimental groups were analyzed using Student's *t*-test. A probability of $P < 0.05$ was considered statistically significant.

Results

Experiment 1

A total of 349 fused oocytes was cultured in six replicates. As shown in Table 1, cleavage, morula and blastocyst rates were similar between starved and non-starved donor cells, or between CDM and CR1aa co-culture. Blastocyst development was approximately 30%. Of the blastocysts produced, 75, 84, and 80.7% were diploid in the starved, non-starved in CDM, and non-starved in the CR1aa co-culture groups respectively (Table 2). The anomalies in the three groups ranged from 16 to 25%. Mixoploids of 2n/3n, 2n/4n, and 2n/3n/4n, and 4n cells were observed among the anomalies.

Experiment 2

As shown in Table 3, 561 reconstructed embryos were randomly cultured in the SCM, BCM, and co-culture systems in seven replicates. Development to the blastocyst stage was similar ($P > 0.05$) in the SCM, BCM, and co-culture groups. Hatching blastocysts were also similar

Table 1 Development of bovine NT embryos using starved and non-starved cumulus cells as donors and cultured in different media.

Co-culture in:	Donor cells	No. of embryos cultured	Cleavage (%)	Morula (%)	Blastocyst (%)
CDM	Starved	158	127 (80.4)	59 (37.3)	44 (27.8)
CDM	Non-starved	100	85 (85)	37 (37)	30 (30)
CR1aa	Non-starved	91	78 (85.7)	36 (39.6)	26 (28.6)

Table 2 Chromosomal composition of blastocysts from starved and non-starved donor cells and cultured in different media.

Co-culture in:	Donor cells	No. of blastocysts analyzed	Blastocysts containing (%):					Total with anomalies
			2n	2n/3n	2n/4n	2n/3n/4n	4n	
CDM	Starved	28	21 (75)	1 (3.5)	2 (7.1)	0	4 (14.2)	7 (25)
CDM	Non-starved	25	20 (84)	1 (4)	2 (8)	1 (4)	1 (4)	5 (16)
CR1aa	Non-starved	26	21 (81)	1 (3.8)	2 (7.6)	1 (3.8)	1 (3.8)	5 (19.3)

Table 3 Development of NT embryos cultured in SCM, BCM, and co-culture.

Culture	No. of embryos cultured	Cleavage (%)	Morula (%)	Blastocyst (%)	Hatching blastocysts (% of blastocysts)
SCM	209	174 (83.3)	67 (32.1)	50 (23.9)	27 (54)
BCM	184	149 (81.0)	76 (41.3)	45 (24.4)	26 (57.8)
Co-culture	168	137 (81.5)	65 (38.6)	50 (30.0)	33 (66)

Table 4 Chromosomal composition of blastocysts derived from SCM, BCM, and co-culture.

Culture	No. of blastocysts analyzed	Blastocysts containing (%):						Total with anomalies	Blastocyst cell no. (n)*	
		2n	2n/3n	2n/4n	2n/3n/4n	4n	Aneuploidy		Diploids	Non-diploids
SCM	41	24 (58.5) ^c	2 (4.9)	7 (17.0)	2 (4.9)	4 (9.7) ^a	2 (4.9) ^a	17 (41.5) ^a	83.3 ± 20.1 ^a (24)	59.2 ± 18.7 ^c (17)
BCM	35	24 (68.6) ^b	2 (5.7)	3 (8.5)	1 (2.8)	3 (8.5) ^b	2 (5.7) ^a	11 (31.4) ^a	86.6 ± 16.7 ^a (24)	57.8 ± 11.8 ^c (11)
Co-culture	42	34 (80.9) ^a	1 (2.4)	4 (9.5)	0	1 (2.4) ^b	2 (4.8) ^a	8 (19.1) ^b	99.8 ± 19.1 ^a (34)	53.3 ± 27.1 ^c (8)

Values with different superscripts within each column except the 'Blastocyst cell no.' column are significantly different ($P < 0.05$). *In the 'Blastocyst cell no.' column, values within each row: a, c $P < 0.01$.

between the treatment groups. The chromosomal composition of blastocysts, however, was significantly different between the SCM, BCM, and co-culture groups. Only 58.5% of blastocysts from the SCM group were diploid, which was significantly lower than the BCM (68.6%, $P < 0.05$) and the co-culture groups (80.9%, $P < 0.01$; Table 4). Aneuploid cells were also among the anomalies observed. Figure 1 shows the various categories of chromosomal composition in NT blastocysts. The cell number of blastocysts in the co-culture group was higher than that observed in CM groups, though the difference was not significant. Considerably fewer ($P < 0.01$) cells were observed in blastocysts with chromosomal anomalies than those with normal diploid compositions (Table 4).

Experiment 3

Based upon the findings in experiment 2 (that blastocysts derived from CM culture possessed more chromosome anomalies than co-culture), experiment 3 was designed to examine whether the diploid blastocyst rate could be improved by exchanging the culture system after the first 72 h of culture. No improvements were observed after exchanges from SCM to CO (SCM–CO) or from CO to SCM (CO–SCM). Blastocyst rates were similar ($P > 0.05$) among the four groups as shown in Table 5. Chromosomal anomalies in SCM–SCM, SCM–CO, and CO–SCM groups were 39.1, 45.8, and 31.8% respectively, which were significantly higher ($P < 0.05$) than that in the co-culture group (Table 6). A 1n/2n mixoploid blastocyst was found in the SCM–CO culture group, where seven identifiable haploid cells and four diploid cells were found out of 121 total cells (Fig. 1 shows the 1n). The cell number in chromosomally abnormal blastocysts was significantly lower ($P < 0.01$) than in diploid blastocysts (Table 6).

Summarizing the results of experiments 1 through 3, the diploid blastocyst rate was significantly higher ($P < 0.001$) in co-culture (120/151, 79.5%) than in CM (62/99, 62.6%) (Table 7). There was no difference in the mitotic index (number of metaphase nuclei/total number of nuclei) between CM culture and co-culture embryos (11.8 ± 5.6 and 12.1 ± 4.7 respectively).

Discussion

No reports have been published comparing methods of preparing CM with cumulus cells for use in culturing bovine NT embryos. Results in this study indicate that CM prepared with bovine cumulus cells, either serum-containing CM, or BSA-containing CM, yielded similar morula and blastocyst development compared with co-culture with bovine cumulus cells (Table 3). There have, however, been numerous studies reporting the incubation of *in vitro* produced (IVP) or *in vivo* embryos in medium conditioned with different cells (Kobayashi *et al.* 1992, 1997, Baranao *et al.* 1997, Liu *et al.* 1998, Joo *et al.* 2001). Cell numbers of blastocysts derived in CM were not different from co-culture groups. Our data support the collective reports of others indicating that CM conditioned by different cells yields similar morula and blastocyst development to the co-culture system, whether the embryo is derived by *in vivo*, IVP, or NT procedures.

Results in this study showed that there were no differences in diploid blastocyst rates between CDM and CR1aa groups, or between starvation and non-starvation groups (Table 2). This suggests that culture medium and donor nuclear cell status do not affect chromosome composition at the blastocyst stage. This conclusion is in agreement with other studies where 82% (Mohamed & Takahashi 2000) and 87% (Booth *et al.* 2003) of

Figure 1 Chromosome spreads of haploid (A, two groups of haploid chromosomes), aneuploid (B, 56 chromosomes), diploid (C and D), triploid (E), and tetraploid (F). (A), (B), (C), (E), and (F) from CM culture and (D) from co-culture. (B), (C), and (D) 1000 × and (A), (E), and (F) 400 ×.

NT blastocysts derived from cumulus cells, 78% from fetal fibroblasts and 88% from adult fibroblasts (Arat *et al.* 2002) were diploid. After culture with CM, however, a significantly higher incidence of chromosomal abnormalities was observed in SCM and BCM groups than in the co-culture group (Table 4). Normal chromosome complement rates did not improve, anomalies were still higher in SCM and changed groups than in co-culture alone in experiment 3 (Table 6). The results of this study suggest

that factors leading to a higher incidence of chromosomal anomalies may be inherent within the CM.

Diploid chromosome rates of NT bovine blastocysts determined by karyotyping are approximately 80% (Mohamed & Takahashi 2000, Arat *et al.* 2002, this study). Similar results were also reported by using fluorescent *in situ* hybridization protocols (Booth *et al.* 2003, Slimane-Bureau *et al.* 2003). Mixoploids of 2n/3n, 2n/4n, and 2n/3n/4n, tetraploids, and aneuploids were observed in

Table 5 Blastocyst development of NT embryos after exchanges from SCM to CO or from CO to SCM 72 h later.

1st culture	2nd culture	No. of embryos cultured	Cleavage (%)	Morula (%)	Blastocyst (%)	Hatching blastocysts (% of blastocysts)
SCM	SCM	115	97 (84.3)	32 (27.8)	23 (20)	9 (39)
SCM	Co-culture	114	97 (85)	34 (29.8)	26 (22.8)	14 (53.8)
Co-culture	SCM	118	101 (85.6)	29 (24.5)	22 (18.6)	5 (22.7)
Co-culture	Co-culture	115	95 (82.6)	41 (35.7)	32 (27.8)	15 (46.9)

Table 6 Chromosomal composition of the blastocysts derived from the exchanged culture system.

Culture groups	No. of blastocysts analyzed	Blastocysts containing (%):						Total with anomalies	Blastocyst cell no. (n)*	
		2n	1n/2n	2n/3n	2n/4n	2n/3n/4n	4n		Diploids	Non-diploids
SCM–SCM	23	14 (60.9) ^b	0	2 (8.7)	4 (17.4)	1 (4.3)	2 (8.7)	9 (39.1) ^a	82.2±19.5 ^a (14)	72.1±19.3 ^b (9)
SCM–CO	24	13 (54.2) ^b	1 (4.2)	2 (8.3)	7 (29.1)	1 (4.2)	0	11 (45.8) ^a	121.2±13.2 ^a (13)	61.5±21.0 ^c (11)
CO–SCM	22	15 (68.2) ^b	0	3 (13.6)	3 (13.6)	0	1 (4.5)	7 (31.8) ^a	85.3±26.5 ^a (15)	58.8±24.2 ^c (7)
CO–CO	30	24 (80) ^a	0	3 (10)	2 (6.7)	0	1 (3.3)	6 (20) ^b	96.2±20.1 ^a (24)	57.1±15.6 ^c (6)

Values with different superscripts within each column except the 'Blastocyst cell no.' column are significantly different ($P < 0.05$). *In the 'Blastocyst cell no.' column, values within each row: a,b $P < 0.05$; a,c $P < 0.01$.

Table 7 Summary of chromosomal composition in blastocysts yielded from CM and the co-culture system.

Culture	No. blastocysts analyzed	Blastocysts containing (%):					Aneuploidy	Total with anomalies (%)
		2n	2n/3n	2n/4n	2n/3n/4n	4n		
Co-culture	151*	120 (79.5) ^a	7 (4.6)	12 (7.9)	2 (1.3)	3 (2.0)	2 (1.3)	31 (20.9) ^c
CM**	99	62 (62.6) ^c	6 (6.0)	17 (17.2)	4 (4.0)	7 (7.0)	4 (4.0)	39 (39.4) ^a

*Data include CDM groups in Table 1. **Does not include SCM–CO and CO–SCM data.

a,c $P < 0.001$.

this study. A majority of the mixoploid blastocysts were diploid–tetraploid, which is consistent with previous reports for NT embryos (Mohamed & Takahashi 2000, Arat *et al.* 2002, Booth *et al.* 2003) and IVP embryos (Hare *et al.* 1980, Iwasaki *et al.* 1992, Mohamed & Takahashi 2000). A haploid/diploid blastocyst was observed in the SCM–CO culture group. Mixoploidy of 1n/2n is usually found in 2-cell (Slimane *et al.* 2000) and in 4- to 32-cell embryos (Iwasaki *et al.* 1992). It has been suggested that the 1n/2n mosaic results from the retained activity of a polar body or is a product of polyspermy (King *et al.* 1981, Iwasaki *et al.* 1992). The mechanism that gives rise to a haploid NT blastocyst is unclear.

Chromosomal analysis of bovine embryos has indicated an overall incidence of abnormalities of 10% in *in vivo*-derived embryos and 15–30% in IVP embryos (Kawarsky *et al.* 1996, Viuff *et al.* 2002). In another study, 20% of *in vivo* bovine blastocysts and 44% of IVP blastocysts had anomalies (Iwasaki *et al.* 1992). The rate of embryos with anomalies in whole blastocysts was significantly higher than in inner cell mass cells only, and most were 2n/4n (Iwasaki *et al.* 1992). *In vivo*, 2n/4n mixoploids have been observed in day 12–18 bovine blastocysts (Hare *et al.* 1980). Results from diploid–tetraploid chimeras have confirmed that even high proportions of tetraploid cells are tolerated, but the tetraploid cells are eliminated in the developing embryo and are preferentially allocated to the extra-embryonic membranes (James *et al.* 1995). The fact that live-born calves do not usually exhibit chromosomal abnormalities suggests that embryos

or fetuses with chromosomal anomalies are eliminated prenatally (King 1990, Kawarsky *et al.* 1996).

The formation of mixoploid embryos is assumed to result from cytokinetic failure, blastomere fusion, endoreduplication, or combinations of these events (King 1990). Tetraploid cells may arise through the fusion of two diploid blastomeres in a growing embryo (King *et al.* 1981) or from nuclear division without cytoplasmic division (Hare *et al.* 1980). Moreover, abnormalities imposed during *in vitro* culture of the embryo may cause abnormal chromosome segregation leading to mosaicism during cleavage, although Schumacher *et al.* (1998) concluded that physical factors associated with *in vitro* culture do not increase DNA ploidy abnormalities in rabbit embryos.

A high incidence of anomalies occurred in the CM groups in this study. The diploid complements were not improved even when a culture-change system was used. In a co-culture system, both the helper cell and the embryo contribute to a dynamic environment that results in increasing concentrations of growth factors and cytokines (Desai & Goldfarb 1998). The rates of diploid blastocysts under co-culture versus non-co-culture conditions (cultured in CR1aa+3% FCS, data not shown) were not different, but both were significantly higher than embryos in the CM group. The presence of serum in CM, that is SCM, caused more anomalies than BCM (Table 4). Coincidentally, both studies reported a high incidence of chromosomal abnormalities in bovine embryos (32–45% in day 5 (Kawarsky *et al.* 1996), and 44% in days 6–8) (Iwasaki *et al.* 1992) cultured in a co-culture system with

cumulus cells (Iwasaki *et al.* 1992) or oviductal epithelial cells (Kawarsky *et al.* 1996) with medium TCM 199. A lower incidence of chromosomal anomalies was reported when cloned embryos were cultured in simple media (Mohamed & Takahashi 2000, Arat *et al.* 2002). Because complex medium contains many components, as does serum, and particularly when the two are used together, it is difficult to identify factors that affect embryonic development and those factors that might alter the genetic composition of the embryo. It becomes even more complicated when a co-culture system is involved and more complex still when considering the potential effects of the NT procedure. All of these factors, particularly when used in an NT culture system, can affect chromosomal behavior and thus lead to chromosomal abnormalities.

The results of this study also showed that the rate of embryo development was significantly affected by the chromosomal complement. Embryos with chromosomal anomalies had decreased development, and cell number was significantly lower than diploid blastocysts in either CM or in co-culture (Tables 4 and 6). Morphologically poor blastocysts tended to have lower cell numbers and exhibited more chromosomal errors (King *et al.* 1987, Kawarsky *et al.* 1996).

In summary, CM has similar effects on the development of bovine NT embryos as the co-culture system. Cumulus cell CM may contribute to an increased incidence of blastocyst chromosomal abnormalities. In turn, chromosomal anomalies may lead to lower cloning efficiency and higher rates of embryonic, fetal, and perinatal mortality.

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