Chromosome Preparation from 2-cell Bovine Embryos derived from Follicular Oocytes Fertilized *in vitro*

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Summary. Methods for collecting cells in metaphase and spreading chromosomes (hypotonic treatment) were investigated in 2-cell bovine embryos derived from *in vitro* fertilization and the sex ratio was studied. The embryos were exposed to 0.08 or 0.16 $\mu\text{g/ml}$ of vinblastin-podophyllotoxin (V-P) for 12 hr at 39°C before being treated with 0.75–1.0% of sodium citrate solution for 15 to 60 min. The exposure to 0.08 $\mu\text{g/ml}$ V-P yielded analyzable metaphases in 69.1% of the embryos prepared, which was contrasted with 42.9% in the exposure to 0.04 $\mu\text{g/ml}$ colcemid for 4 hr. A hypotonic treatment with 1.0% sodium citrate for 60 min was recommended to obtain a high quality of metaphase spread. Chromosomal sexing was made for 61 embryos with a sex ratio of 63.9% males.

KEY WORDS: 2-CELL BOVINE EMBRYOS, CHROMOSOME, IN VITRO FERTILIZATION.

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Chromosomes of early embryos have been analyzed for estimating the sex ratio and incidence of chromosome anomalies at early cleavage stages in mice (Kaufman, 1973; Kasai *et al.*, 1981; Yoshizawa *et al.*, 1985), sheep (Long and Williams, 1980) and pigs (Long and Williams, 1982). In bovine embryos, chromosome studies have been centered on the preimplantation stages (Hare *et al.*, 1976; Wintenberger-Torres and Popescu, 1980; Hishinuma *et al.*, 1984), with a few reports being concerned with the 1- to 2-cell stages of embryos (King *et al.*, 1979, 1981). In these and other related studies, Tarkowski's air-drying method for mouse eggs has been used with some modifications to adapt to different sources and stages of embryos. When we work in 1- to 2-cell embryos, however, difficulty still remains that the limited number of their component cells, hence the scarcity of dividing cells, lowers analyzable rate.

In the present study, we attempted to increase metaphase figures in 2-cell bovine embryos by the use of vinblastin-podophyllotoxin (V-P) as a

mitosis-arresting agent instead of colchicine or colcemid. The way of hypotonic treatment was also reexamined because this procedure involves the quality of metaphase spreads. Follicular oocytes were used as a source of embryos in view of the increasing interest in utilizing them.

Materials and Methods

1. Preparation of 2-cell embryos

Bovine ovaries were obtained at a slaughterhouse and follicular oocytes were collected by the method described in a previous paper (Iwasaki *et al.*, 1987). To achieve maturation, the follicular oocytes surrounded by complete cumulus cells were cultured in HEPES-buffered TCM 199 (GIBCO) containing 10% fetal calf serum, 1 $\mu\text{g/ml}$ of estradiol-17 β , 10 $\mu\text{g/ml}$ of luteinizing hormone (Burns-Biotec) and antibiotics for 26 hr at 39°C in an atmosphere of 5% CO₂–95% air. Frozen semen from a Japanese Black bull was thawed and washed with 10 mM caffeine-sodium benzoate in a chemically defined medium by

Brackett and Oliphant (1975), but without bovine serum albumin, and adjusted to a concentration of 25×10^6 sperm/ml. Semen was mixed with 20 mg/ml of bovine serum albumin (Sigma) and preincubated for 5 hr at 39°C. The matured oocytes were incubated *in vitro* with the capacitated spermatozoa for 6 hr. Fertilized eggs were transferred to a medium for development (HEPES-buffered TCM 199 containing 10% steer serum, 0.5 mM sodium pyruvate, 0.02 mM sodium lactate and antibiotics). Two-cell embryos were obtained about 28 hr after the beginning of sperm-egg incubation.

2. Preparation of chromosome slides

The majority of the 2-cell embryos were further cultured in TCM 199 medium containing 0.08 or 0.16 $\mu\text{g/ml}$ for both of V-P for 12 hr. For comparison, some other embryos were cultured in TCM 199 medium containing 0.04 $\mu\text{g/ml}$ of colcemid for 4 hr at 39°C. Subsequently, embryos were transferred to phosphate buffered saline (PBS) and the zona pellucida was weakened by treating embryos with 0.5% Actinase E (Kaken Kagaku Co. Ltd) in PBS for 40 sec. Then they were placed in hypotonic solution (0.75–1.0% of sodium citrate) for 15, 30 or 60 min at room temperature. The first fixation was made by transferring the embryos to a fixative solution consisting of acetic acid, methanol and distilled water (8:5:15) for 3–10 min. The second fixation was carried out by placing them in a mixture of acetic acid-methanol (1:3) for 20–40 min. The embryo was transferred to a slide glass and spreading was accomplished by blowing on the slide. The preparations were inspected with a phase contrast microscope and stained with 4% Giemsa (Merk) for 4 min. For good metaphase

spreads, chromosomes were counted and the sex was diagnosed based on the observation of large (X) and small (Y) submetacentric chromosomes.

Results and Discussion

1. Effect of V-P treatment

The effects of V-P and colcemid in arresting cleavage division at metaphase are summarized in Table 1, where the degrees of the effectiveness are shown in terms of the analyzing rate which means the percentage of analyzed embryos to the embryos prepared. Out of 165 embryos treated with 0.08 $\mu\text{g/ml}$ of V-P for 12 hr, 114 showed metaphase spreads with countable chromosomes. Thus the analyzing rate was 69.1%. Of these, 63 embryos (55.3%) had two sets of normal diploid metaphase spread, so that the relative number of analyzable cells was considerably increased by the V-P treatment. A high concentration (0.16 $\mu\text{g/ml}$) of V-P did not necessarily improve the analyzing rate (61.1%). With 0.04 $\mu\text{g/ml}$ of colcemid, analyzing rate was 42.9%, which was comparable with 35–45% obtained by Kasai *et al.* (1981) in mouse embryos treated with colcemid at the second cleavage division. The analyzing rate in the V-P treatment was significantly higher than that in the colcemid treatment ($P < 0.05$). The duration of V-P treatment was 12 hr, being three times longer than that of colcemid treatment. This long term of exposure to V-P may increase the number of cells arrested at metaphase. Furthermore, quite unlike colcemid, V-P did not cause over-contraction of chromosomes even after such a long term treatment. These may account for the higher analyzing rate in the V-P treatment.

Table 1. Chromosomal analyzing rates in 2-cell bovine embryos treated with vinblastin-podophyllotoxin (V-P) and colcemid

Chemicals	Concentration ($\mu\text{g/ml}$)	No. of embryos processed (A)	Analyzed embryos*				Analyzed rate (B/A, %)
			2N/-	2N/2N	Abn.	Total (B)	
V-P	0.08	165	31	63	20	114	69.1 ^{a**}
	0.16	18	3	7	1	11	61.1 ^{ab}
Colcemid	0.04	14	5	1	0	6	42.9 ^b

* 2N/-: with one diploid metaphase, 2N/2N: with two diploid metaphases, Abn.: with abnormal number of chromosomes including 2N+1, 3N and 2N/4N.

** Figures with different superscript letters were significantly different at 5% level.

Table 2. Sexing rates in 2-cell bovine embryos classified according to various conditions of hypotonic treatment

Sodium citrate		2-cell embryos		Embryos with normal diploid			Sexing rate (%)
Conc. (%)	Time (min)	Processed	Analyzed	Female	Male	Undefined	
1.0	60	84	52	7	18	19	56.8
1.0	30	12	8	2	1	2	60.0
0.95	60	16	9	3	1	3	57.1
0.9	30	23	16	3	4	7	50.0
0.9	15	13	13	1	6	5	58.3
0.85	60	7	3	0	2	1	66.7
0.8	30	6	6	2	2	0	100.0
0.8	15	13	11	2	3	5	50.0
0.75	60	10	7	2	2	1	80.0
Total		184	125	22	39	43	58.7

*: No. of male and female embryos/No. of embryos with normal diploid metaphase.

Fig. 1. A male karyotype from 2-cell bovine embryos treated with 0.08 $\mu\text{g}/\text{ml}$ vinblastin-podophyllotoxin for 12 hr followed by hypotonic treatment with 1% sodium citrate for 60 min, Giemsa stain.

2. Effect of hypotonic treatment on sexing rate

We obtained 125 analyzable embryos after the treatment with 0.08 and 0.16 $\mu\text{g}/\text{ml}$ of V-P (Table 1). Of these 104 embryos with normal diploid metaphases were devoted to cytological sexing, of which 61 could be successfully sexed, with a sexing rate of 58.7% (Table 2). Sexing rates ranged from 50 to 100%, when classified according to various combinations of the concentration of sodium citrate solution and the

duration of the treatment. Although a high sexing rate was obtained by a combination of 0.8% for 30 min or of 0.75% for 60 min, such a prolonged treatment with lower concentration caused over-scattering of chromosomes. Furthermore, several sorts of chromosome deformation, such as slenderness, distortion, blur or interior gaps, were also observed. Even with a concentration of 0.8 or 0.9%, the treatment for a short period of time (15 min) caused insufficient spreading of chromosomes.

In many cases of preparations, a combination of 1.0% for 60 min was associated with good spreading of metaphase chromosomes (Fig. 1).

Tarkowski (1966), in his original method, treated mouse eggs with 1% sodium citrate for 5–15 min, recommending that the condition should be changed according to the ages and size of embryos. According to King (1984), 12- to 15-day bovine embryos have been preferably treated with 0.88% Na citrate for 3–5 min.

Our results showed that in the preparation from 2-cell bovine embryos the duration of hypotonic treatment was desirable to be prolonged up to 30–60 min.

3. Sex ratio

Of 61 embryos for which the sex was determined, 22 (36.1%) were found to be female and 39 (63.9%) male (Table 2). The sex ratio of mouse eggs at the first cleavage division was 50.4% by Kaufman (1973) and 43.5% (n=767) by Yoshizawa *et al.* (1985). In cattle, Bouters *et al.* (1980) reported that the sex ratio was 58% males (n=87) among 12- to 14-day-old embryos. It was reported that among abortuses and still-born calves 60% were males (Powell *et al.*, 1975). Our result was also suggestive of high embryonic sex ratio in cattle.

Some chromosome anomalies were observed, which will be described in detail elsewhere.

In conclusion, we recommended the following method for preparing analyzable metaphases from 2-cell bovine embryos. That is, the embryos harvested 28 hr after the beginning of sperm-egg incubation are treated with V-P (0.08 µg/ml for both) for 12 hr and subsequently treated with 1.0% of sodium citrate for 60 min.

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体外受精由来ウシ2細胞期胚の染色体標本作製法の検討

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体外受精により作出したウシ2細胞期胚を用い、染色体標本作製のための分裂中期停止及び低張処理条件を検討した。同時に染色体検査により性判別を行った。

分裂中期核板検出率は、0.08 $\mu\text{g/ml}$ の Vinblastin-Podophyllotoxin, 12時間処理区で高く (69.1%), この濃度を2倍に増しても検出率の増加はみられなかった。Colcemid (0.04 $\mu\text{g/ml}$) 4時間処理区での検出率は42.9%で低かった。クエン酸ナトリウムによる低張処理は濃度 (0.75~1.0%), 時間 (15, 30, 60分間) とともに性判別率に影響はなく, 中期核板像を有する125の胚のうち,

61個の胚で性判別が可能であった。内訳は雌: 22 (36.1%), 雄: 39 (63.9%) であった。低張処理濃度の低下に伴い染色体は分散し, 間隔が広がり, 染色体の辺縁が滲んで輪郭が不鮮明になる傾向がみられた。濃度は0.9~1.0%でも処理時間を短縮した場合には, 染色体の分離が不十分で, 判別が困難であった。

以上の結果より, ウシ2細胞期胚の分裂中期像を得るには, 媒精28時間後の胚を Vinblastin-Podophyllotoxin (0.08 $\mu\text{g/ml}$) で12時間処理し, 1%クエン酸ナトリウムで60分間低張処理する方法が最適と考えられた。