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Transition of Cleavage Divisions during In Vitro Development of Bovine Embryos

Hitoshi Ushijima[1, 2]*, Kiyoshi Akiyama[3] and Toshio Tajima[2]

[1]Nippon Veterinary and Life Science University, Musashino 180-8602, Japan
[2]Chiba Prefectural Livestock Experimental Station, Yachimata 289-1113, Japan
[3]Kanagawa Prefectural Livestock Industry Technology Center, Hongo 3750, Ebina 243-0417, Japan

Abstract: *Bovine in vitro fertilized (IVF) embryos were cultured in either CR1aa (CR1) or TCM-199 (TCM) medium and compared for their daily development based on the number of cleavage divisions (CDN), as calculated from the total cell numbers of the embryos. Embryos with advanced developmental stage and higher morphological quality were selected for use in the experiment. The relation between the CDN and the number of embryonic days after IVF in both groups showed a linear correlation; no significant difference ($P > 0.05$) was found between the two groups. However, CDN on days 3 and 8 after IVF in the CR1 group did not increase, which suggests that transient developmental arrests occurred at these stages. In contrast, embryos in the TCM group showed a transient developmental arrest 3 d after IVF, but CDN increased regularly with age in days at later stages of culture with 100 μM β-mercaptoethanol. A significant difference was found between the regression lines of the two groups during 5–9 d after IVF ($P < 0.001$). Consequently, numerical analysis of embryonic development in terms of CDN enabled objective evaluation of the developmental progress of bovine IVF embryos.*
Key words: *Bovine IVF embryo, β-mercaptoethanol, Cleavage division, Embryonic development*

Introduction

Embryonic environments such as culture conditions and media have been examined to find new ways of enhancing development of *in vitro* production (IVP) of embryos [1–3]. These IVP systems, *in vitro* maturation/ *in vitro* fertilization/ *in vitro* culture (IVM/IVF/IVC), have been used extensively to produce calves for the livestock industry [4, 5]. However, compared to *in vivo*-collected (VIVO) embryos, these IVP embryos have lower developmental ability after cryopreservation, manipulation, and transfer to recipients [6–9]. Therefore, continual improvement of IVP systems is required [10–12].

Preimplanted embryos grow by self-replicating blastomeres. A bovine embryo on days 6–7 after ovulation is known to usually have over 100 cells [13]. The total cell number (TCN) is commonly used as an indicator of embryo quality for evaluating and comparing the development potential of bovine IVF embryos among different IVP methods [1, 4, 6] or for evaluating the viability of frozen-thawed embryos [8, 9]. The ratio of the apoptosis index within an embryo [14] or the inner cells/outer cells ratio of a blastocyst [15, 16] is also used to describe the embryonic quality. As another approach, the number of cleavage divisions (CDN) is calculated using logarithmic transformation of TCN; the developmental progress or the segmental speed is expressed as a function of embryonic day and CDN [17, 18]. We recently used this method to compare the development of IVF embryos that had been cultured in TCM-199 with a cumulus cell monolayer for 9 d (co-cultured method) for development of VIVO embryos. The results show that developmental arrest occurred in IVF embryos, but not in VIVO embryos [19]. A criterion based on CDN presents the possibility of evaluating embryonic development from a new perspective.

For this study, we used two different culture media, both of which are highly efficient methods in IVP systems and which are used for calf production on a commercial basis [4, 5, 20, 21]. We compared the developmental efficiency of the culture media using embryonic age and CDN.

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*To whom correspondence should be addressed.
e-mail: ushijima@nvlu.ac.jp

Materials and Methods

Production of IVF embryos

Cumulus-oocyte complexes (COCs) from ovaries of slaughtered Holstein cows were collected according to the protocol described by Shino *et al.* [20]. In brief, oocytes with follicular fluid were aspirated from small follicles (2–5 mm diameter) at the ovarian surface using a disposable syringe and a needle. The COCs were washed twice with 25 mM Hepes-buffered TCM-199 with Earle's salt (Gibco Invitrogen) supplemented with 5% (v/v) heat-treated fetal calf serum (FCS, Gibco BRL, Grand Island, NY, USA), 50 μg/ml streptomycin, and 100 μg/ml penicillin-G (Meiji Seika, Tokyo, Japan) under mineral oil (Sigma-Aldrich, St. Louis, MO, USA). In a single drop of the same medium (TCM), 50–60 COCs were cultured at 38.5°C in an atmosphere of 3% CO_2 in air for 20 h [19]. The IVM oocytes were stored in TCM in 5-ml plastic tubes and transported within 1 h from the slaughterhouse to the laboratory in a container maintained at 38°C (FHK, Tokyo, Japan) [5].

Frozen Holstein semen was immersed in a 38°C water bath for 15 s, suspended in 10 ml of BO medium containing 5 mM caffeine (Sigma) and washed twice by centrifugation at 500 × g for 5 min to remove cryoprotectants. Spermatozoa were resuspended in the BO medium containing 5 mM caffeine, 5 μg/ml heparin (Novo-heparin; Kodama, Tokyo, Japan) and 10 mg/ml bovine serum albumin (BSA, fraction V; Sigma) [5, 6]. The final concentration of spermatozoa in the IVF medium was 5–10 × 10^6/ml. The IVM oocytes were subsequently fertilized *in vitro* for 5 h. After IVF, oocytes were removed from the cumulus cell masses by pipetting and were cultured at 38.5°C in either of two culture media: CR1aa (CR1) or TCM. For CR1 culture [3, 4], embryos were cultured in a 100-μl drop of CR1 + 5% FCS for 5 d in an atmosphere of 5% CO_2 in air. The medium was exchanged with CR1 + 10% FCS 5 d after IVF, and culture was continued for 4 d under identical atmospheric conditions. For culture in TCM, embryos were co-cultured in maturation medium under an atmosphere of 3% CO_2 in air [19] for 5 d. Then, embryos were transferred to a 100-μl drop of TCM + 10% FCS supplemented with 100 μM β-mercaptoethanol (BME, Sigma) [20, 21] and were cultured under an atmosphere of 5% CO_2 in air for 4 d. For comparing the *in vitro* development of the two groups, embryos at the stage of zygote to hatched blastocyst, 0–9 d after IVF, were selected for the following experiments.

Selection of embryos

Embryos of many types at different developmental stages and various morphological qualities were produced using our IVM/IVF/IVC system [20]. The TCN of embryos corresponds exactly to the embryonic developmental stage [16] if high-quality embryos are selected according to their morphology. Therefore, embryos were classified into five quality groups according to the embryo evaluation method [22] using an inverted phase contrast microscope (IMT 10; Nikon, Tokyo, Japan). We selected 1–2 embryos from each batch as excellent quality embryos according to their advanced developmental stage and higher morphological quality.

Cell count of embryos and calculation of cleavage division (CD)

Embryos were fixed using Tarkowski's air-dry method [23] with slight modification and were stained with 5% Giemsa stain to allow counting of cells. In brief, embryos were hypotonically treated with 0.9% sodium citrate solution for 5 min at room temperature and were then fixed onto a spot plate (Corning Labware and Equipment, Corning, NY, USA) for 1 min using an ice-cooled fixative consisting of methanol, acetic acid, and distilled water (3:2:1). Embryos were then mounted on a glass slide using a small amount of fixative, air-dried for 1 h at room temperature, and stained with Giemsa stain (Gibco-Invitrogen, Tokyo, Japan) for 10 min [19]. The TCN of the embryos was counted under a microscope at 400 × magnification. The CDN of the embryos was calculated as $\log_2$ of the TCN. The relation between CDN and embryonic age in days was studied.

Statistics

Developmental rates of embryos between the groups shown in Table 1 were evaluated using the chi-square test. Student's *t*-test was used to compare the mean CDN of embryos. A probability of less than 0.05 was inferred to be significant. For the regression line of the CDN and embryonic age, the slope indicates the embryonic cleavage speed. The regression line slopes were compared using covariance analysis [19].

Results

Table 1 summarizes the results of *in vitro* development of embryos cultured in CR1 or TCM. Embryos in both groups developed to the two-cell stage 1 d after IVF and subsequently progressed into the

Table 1. *In vitro* development of IVM/IVF bovine embryos

Culture medium[1)]	No. cultured	No. (%) of embryos developed to			
		2-cell	Blastocyst	High quality blastocyst	Hatched blastocyst
CR1	447	365 (82)	135 (30)	62 (14)	67 (15)
TCM	348	289 (83)	106 (30)	44 (13)	60 (17)

[1)]CR1: CR1aa + 5% FCS for 5 d followed by CR1aa + 10% FCS for 4 d. TCM: TCM199 + 5% FCS with cumulus cells for 5 d followed by TCM199 + 10% FCS supplemented with 100 μM β-mercaptoethanol for 4 d.

Table 2. Number of cleavage divisions (CDN) of bovine embryos cultured in CR1 and TCM for 0–9 d after *in vitro* fertilization

Day	CR1		TCM	
	No. of embryos examined	CDN of embryos (mean ± S.D.)	No. of embryos examined	CDN of embryos (mean ± S.D.)
0	10	0 ± 0	10	0.0 ± 0.0
1	12	1.1 ± 0.2 *	10	1.1 ± 0.2 *
2	10	3.9 ± 0.1 a*	10	3.5 ± 0.4 c*
3	10	3.9 ± 0.1 b	10	4.4 ± 0.3 a*
4	10	4.2 ± 0.2 d*	10	4.6 ± 0.4 a
5	10	5.9 ± 0.3 a*	10	5.6 ± 0.3 d*
6	9	6.7 ± 0.3 *	8	6.8 ± 0.2 *
7	10	7.3 ± 0.2 b**	10	7.6 ± 0.2 a*
8	9	7.7 ± 0.1 d*	10	8.3 ± 0.2 a*
9	10	8.6 ± 0.2 b*	10	9.0 ± 0.2 a*

*, **: Comparison of CDN with the preceding stage: *P < 0.001, **P < 0.01. Comparison between CR1 and TCM: a>b (P < 0.001); a>c (P < 0.01), and a>d (P < 0.05).

blastocyst stage 7 d after IVF. The two groups' rates of development into the blastocyst stage and high quality blastocysts with no degenerated blastomeres were not significantly different (P > 0.05).

The progress of CDN during *in vitro* development of IVF bovine embryos is presented in Table 2. The CDN in the CR1 group increased irregularly with embryonic age, with the terms of the 2–4th CDs at 1–2 d after IVF being shorter, and those of the 4th CD at 2–3 d and the 8th CD at 7–8 d being longer. Similarly, the CDN of embryos in the TCM group also changed irregularly, with the term of the 2nd–4th CDs at 1–2 d after IVF being shorter, and that of the 5th CD at 3–4 d after IVF being longer. In this group, however, the CDN of embryos at 5–9 d after IVF increased regularly with embryonic age.

Figure 1 depicts the regression equation lines of the CDN of the IVF embryos in each group. The regression equation of the CR1 embryos 0–9 d after IVF was linear, with a high correlation (y = 1.05x, r = 0.96). The slope in the CR1 group was not significantly lower (P > 0.05) than that of the TCM group. However, a significant intergroup difference (P < 0.001) was found in the slope of the regression lines 5–9 d after IVF.

Discussion

Blastomeres in VIVO bovine embryos cleave once a day up to 9 d after ovulation [19], with the proviso that the 1st CD shows a tendency to be slower than 1 d after ovulation, but 2nd to 3rd CDs on days 2–3 tend to occur more quickly [24, 25]. In this experiment, the CDN of IVF embryos cultured in CR1 or TCM media was also expressed as a linear function of embryonic age with a high correlation. However, the developmental speed of the IVC embryos changed irregularly. The IVP embryos cultured in CR1 or TCM media showed improvements in CDN transitions compared to those produced using co-culture methods, but were not comparable to those of VIVO embryos [19] (data not shown).

The developmental speed of bovine IVF embryos is arrested during the genetic shift from the maternal to the embryonic genome at the 3rd–4th CDs [13, 24–26]. In this experiment, the IVF embryos in both groups reached the 2nd CD (3–4 cells) at 1 d and the 4th CD (9–16 cell) at 2 d after IVF, which indicates a passage through the genomic shift without difficulty, and a faster transition than that shown by the VIVO embryos [19]. We also observed developmental arrest at the 5th CD at

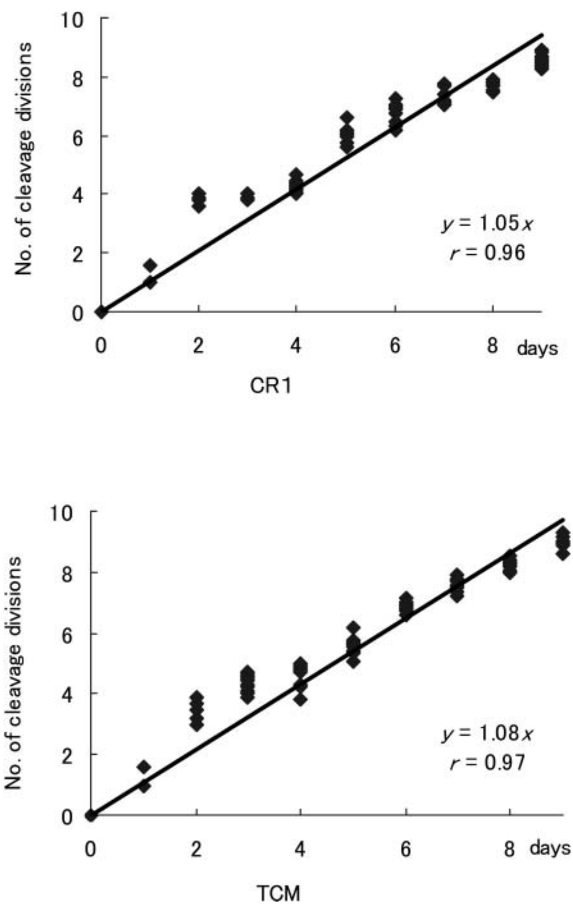

Fig. 1. Transition of cleavage divisions of bovine embryos cultured in the CR1 or TCM medium for 0–9 d after IVF. No significant difference was found between the two regression lines.

2–3 d after IVF in the CR1 group, and at the 6th CD at 3–4 d after IVF in the TCM group, which was not apparent during the development of VIVO embryos [18, 19]. Rapid cleavage and subsequent developmental arrest in IVC embryos have been reported [4, 9, 15, 16]. It remains unclear why this occurs, but we speculate that it is a characteristic mode of development for embryos cultured under suboptimal IVC conditions. Suboptimal IVC conditions are also inferred to adversely affect gene expression in embryos, since lack of gene expression has been reported in IVF embryos [24, 25].

The CR1 medium reportedly produces higher quality embryos than the co-culture method [3, 4]. Furthermore, in the present study, embryonic development as expressed by the relation between the CD and age in days for the embryos cultured in CR1 was faster than that of the co-culture method ($y = 0.83x + 1.02$) described in our previous report [19]. Unlike VIVO embryos, which typically undergo the 9th cleavage 9 d after ovulation, embryos cultured in CR1 showed a developmental arrest 7–8 d after IVF, similar to co-cultured embryos in TCM media [19]. This finding indicates that the CR1 medium reduced embryonic viability and that the conditions were suboptimal for additional embryonic development. In contrast, the CDN of embryos cultured in BME-supplemented TCM increased once daily during the last half of the culture, which is consistent with the developmental progress of VIVO embryos, but with a statistically significant difference (data not shown). Unlike CR1 embryos, embryos cultured in BME-supplemented TCM media showed no developmental arrest in the later stages of culture; the CDN increased regularly with embryonic age. This normal developmental speed, we suggest, is probably the result of BME supplementation rather than the co-culture method [19], as discussed below.

Cysteine and cystine are contained in the TCM medium to support synthesis of glutathione [27], which plays an important role in preventing intracellular oxidative damage [21]. As a thiol compound, BME promotes cysteine and cystine uptake into blastomeres, and enhanced bovine embryonic development subsequent to addition of BME to the culture medium has been reported [5, 21, 27]. A series of biochemical reactions induced by BME is thought to facilitate late stage embryonic development (blastocyst expansion and the hatch from the zona pellucida) by protecting the embryos from oxidative stress, the major cause of developmental arrest [21]. In the present study, the developmental speed of embryos was restored to that of normal bovine embryos when cultured in BME-supplemented media during the later stages of culture. Nedambale *et al.* [28] reported that BME supplementation to CR1 medium enhances embryonic viability. Therefore, BME supplementation appears to be effective not only with TCM but also with media of other types. However, other methods such as addition of antioxidant agents instead of BME [29], *in vitro* culture condition with serum-free media under the regulation of O_2 concentration [11, 27, 30], and the removal of the target of oxygen radicals from the cytoplasm [9, 29] also enhance the embryonic viability. It is a matter of keen interest whether these treatments can restore the developmental speed of IVC embryos.

In conclusion, numerical analysis of embryonic development in terms of CDN enables us to evaluate the embryonic development more objectively. We found

that two culture methods, which had been thought to be similar in IVP efficiency, exhibited different embryonic development as evaluated by the increase of CDN with embryonic age. The developmental speed of IVP embryos was improved by the addition of BME during the latter half of culture. Cleavage occurred once a day, as in VIVO embryos, suggesting that BME prevented developmental arrest in embryos, which has been generally associated with the co-culture and CR1 methods. Future studies should evaluate the developmental ability to term and cryotolerance of blastocysts obtained from this improved IVP method, with the ultimate aim of providing VIVO level quality.

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