Research Article

Efficient Production of Bovine Tetraploid Embryos by Electrofusion of *In Vitro* Produced Two-Cell Stage Embryos

Er-Dan Wang[1,2#], Seok-Hwan Song[1#], A-Na Ha[1], Sang-Ryeul Lee[3], Kyeong-Lim Lee[1], Jae-Ik Lee[1], Zhongde Wang[3], Xian-Feng Yu[4], Wen-Fa Lv[2*] and Il-Keun Kong[1,5*]

[1]*Department of Animal Science, Division of Applied Life Science (BK21 Plus), Gyeongsang National University, Jinju 660-701, Gyeongnam Province, Republic of Korea*
[2]*Department of Animal Science, Division of Animal Reproduction and Breeding, Jilin Agricultural University, 130118, Changchun, Jilin Province, Republic of China*
[3]*Department of Animal, Dairy, and Veterinary Sciences, Utah State University, Logan, UT 84322-4815, USA*
[4]*College of Animal Sciences, Jilin University, Changchun 130-062, China*
[5]*Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 660-701, Gyeongnam Province, Republic of Korea*
[#]*Authors contributed equally to this work*

Abstract

Tetraploid complementation has been used to improve the production of cloned animals. The main objective of this study was to improve the efficiency of bovine tetraploid embryo production and the development potential of bovine tetraploid embryos into blastocysts. We assessed early embryonic cleavage timing and established the defined time quantum to collect synchronous 2-cell stage bovine embryos for electrofusion. Different electrofusion protocols were also tested. The second aim was to monitor ploidy transition by fluorescence visualization to shed lights on the nuclear fusion process of the cytoplasmic membrane-fused 2-cell stage bovine embryos. Karyotypes of day 8 blastocysts (day 0: day of electrofusion) were determined by karyotyping analysis. We found that electrofusion of *in vitro* produced bovine 2-cell stage embryos results in both tetraploid and diploid embryos and that blastocyst formation rates from fused 2-cell stage embryos are affected by the number of electrofusion pulses and the timing of how soon the 2-cell stage embryos are formed post insemination. We identified two distinct nuclear configurations after electrofusion of 2-cell stage embryos, each of which is uniquely related to the formation of tetraploid or diploid embryos. Based on the observation that tetraploid and diploid embryos derived from fused 2-cell stage embryos undergo different timings to become 2-cell stage embryos again, diploid and tetraploid 2-cell embryos can be readily separated after electrofusion. Finally, our study established an experimental protocol for the effective production of bovine tetraploid embryos by electrofusion of 2-cell embryos.

Keywords: Tetraploid; Cattle; Electrofusion; Karyotype assessment

Introduction

Cloning by somatic cell nuclear transfer (SCNT) has been used to successfully produce animals from many different mammalian species such as cattle. However, developmental abnormalities can arise during pregnancy and/or postnatal, limiting the widespread application of this technology [1-3]. Tetraploid embryos are used in the production of transgenic mice as tetraploid complementation with mouse embryonic stem (ES) cells increases the success rate of cloning [4-9]. Through this technology, full-term development of tetraploid embryos following injection of diploid ES cells or induced pluripotent stem (iPS) cells can be achieved whereby the entire embryo proper is of ES- or iPS-origin and the extra embryonic tissues are from the tetraploid embryo [5,7,9-13].

Several approaches have been employed to produce mammalian tetraploid embryos *in vitro* [14]. The first approach is to transfer an embryonic nucleus into a fertilized egg, which results in the production of a tetraploid embryo [15]. It is also possible to produce parthenogenetic tetraploid embryos by inhibiting the formation of both first (1st PB) and second polar bodies (2nd PB) with cytochalasin B or other cytokinesis-inhibition drugs during parthenogenetic activation of oocytes [16,17]. The third method is to fuse two diploid blastomeres at the 2-cell stage embryo with either polyethylene glycol (PEG) [18,19], inactivated Sendai virus [20] or by electrofusion. Among these fusion methods, electrofusion is the most widely used tool for the production of tetraploid embryos [1,21,22] as it is safer than the chemical- or virus-mediated methods and more convenient and economical than microsurgical injection [14].

To date, the generation of bovine tetraploid embryos by electrofusion resulted in extremely low efficiency. Previous studies showed that, while the fusion rates of 2-cell stage bovine embryos are comparable to that observed in the mouse, the status of tetraploidy of the blastocysts developed from the fused 2-cell stage bovine embryos was not investigated [23]. We previously showed that over 90% of the fused 2-cell stage embryos reversed back to diploidy when examined at the blastocyst stage. Through optimizing the electrofusion parameters, such as the field strength of the electric pulse, the pulse duration, and the number of pulses, Curnow et al. [24] obtained a success rate of 12.5% by analyzing chromosome in day 7 blastocysts. This tetraploidation rate at the blastocyst stage is so far one of the highest reported in bovine. However, this tetraploidation rate is too low for practical applications. Consequently, a reliable protocol for more efficiently producing bovine tetraploid embryos *in vitro* is highly desirable.

In the present study, the main objective was to improve the efficiency of bovine tetraploid embryo production by electrofusion of 2-cell stage embryos and for improving the development rate of bovine tetraploid

embryos into blastocysts *in vitro*. The second aim was to monitor ploidy transition by fluorescence visualization to shed lights on the nucleus restructuring process of fused 2-cell stage bovine embryos.

Materials and Method

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise mentioned. Care and use of animals was conducted in accordance with Gyeongsang National University guidelines for the use of laboratory animals (Approval No. GAR-110502-X0017).

Retrieving of cumulus-oocyte complexes (COCs)

Bovine embryos were produced *in vitro* as previously described [25]. In brief, ovaries were obtained from Korean native Hanwoo cows at a local abattoir, and transported within 2 h to the laboratory in physiological saline (0.9% [w/v] NaCl) held at 35–37°C. Immediately after arrival, ovaries were washed with fresh Dulbecco's PBS, and COCs were retrieved. COCs were recovered from 2- to 8-mm diameter follicles using an 18 G needle attached to a suction pump. Only COCs having more than three layers of compact cumulus cells were collected, placed in HEPES buffer and examined under a stereomicroscope. After selection of COCs, they were washed thrice with TL-HEPES medium (114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM NaH_2PO_4, 10 mM $C_3H_5NaO_3$, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 1 μl/ml phenol red, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin).

In vitro maturation (IVM)

COCs were cultured in maturation medium as previously described [26]. In brief, collected COCs (n=60 COCs for each group) were washed thrice in maturation medium (TCM-199) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 μg/ml estradiol-17β, 10 μg/ml FSH, 0.6 mM cysteine, and 0.2 mM $C_3H_5NaO_3$. A group of 60 COCs were cultured in a well of 4-well dish (Nunc Thermo Fisher Scientific, Inc., Roskilde, Denmark) containing 700 μl IVM medium for 22 to 23 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

In vitro fertilization (IVF) and in vitro culture (IVC)

After IVM, COCs were fertilized with thawed Hanwoo bull sperm. Thawing was performed with a water bath set at 36°C for 1 min. Sperm was washed with Dulbecco's PBS (D-PBS), followed by centrifugation at $750 \times g$ for 5 min at room temperature. The pellet was diluted with 500 μl heparin (20 μg/ml) in IVF medium (Tyrodes lactate solution supplemented with 6 mg/ml BSA, 22 μg/ml $C_3H_5NaO_3$, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin) and incubated at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 15 min so that the sperm could acquire fertilizing capacity. Spermatozoa, which were presumed to be capacitated, were diluted in IVF medium at $1–2 \times 10^6$ spermatozoa/ml. Matured oocytes were transferred into IVF medium (700 μl) containing sperm and incubated for 18–20 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

After 20 h, cumulus cells were removed by pipetting with TL-HEPES buffer in a 35-mm dish (SPL Life Sciences Co. Ltd., Gyeonggi-do, Republic of Korea). Denuded zygotes were cultured into a 4-well containing 700 μl CR1-aa medium supplemented with 44 μg/ml $C_3H_5NaO_3$, 14.6 μg/ml glutamine, 10 μl/ml penicillin–streptomycin, 3 mg/ml BSA, and 310 μg/ml glutathione for 3 days (IVC-I).

Early embryonic cleavage time and proper time quantum for collecting good quality 2-cell stage embryos for electrofusion

Embryo cleavage timing (i.e., the first and second cell cycles) was analyzed every 2 h from 22 to 38 h after IVF. Bovine 2-cell embryos were divided into two categories according to cleavage timing. Embryos were considered early cleaved if they reached the 2-cell stage at 26–30 h after IVF. The second group was collected from 30 to 36 h after IVF and considered as late cleaved embryos. Only good quality 2-cell stage embryos were used for electrofusion and embryo culture. Embryo quality was assessed according to Wright and Ellington [27], and good quality embryos were spherical and symmetrical with cells of uniform size. The bad quality embryos have irregular shapes and non-homogenous cytoplasm.

Electrofusion of bovine two-cell stage embryos

Electrofusion was performed immediately after retrieval of good quality 2-cell stage embryos. Embryos were first rinsed in washing solution (electrofusion solution: IVC-I, 1:1) and then in electrofusion solution. A group of ten embryos was transferred and positioned between two parallel stainless-steel electrodes within a chamber (1 mm gap cuvette; NEPAGENE Co, LTD, Chiba, Japan) in the electrofusion system (Model LF101; NEPAGENE Co, LTD, Chiba, Japan). The electrofusion solution was composed of 0.3 M mannitol, 0.1 mM $MgSO_4$, and 0.05 mM $CaCl_2$ supplemented with 0.01% BSA (w/v). A single pulse of 0.75 kV/cm (DC) for 60 μs and two pulses of 1.0 kV/cm (DC) for 25 μs were applied to find out the optimal electrofusion parameters. After electrofusion, embryos were rinsed thrice in IVC-I medium and then cultured in IVC-I medium at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Unfused embryos were removed at 2 h after electrofusion. On the other hand, embryos fused into an intact single-cell embryo were cultured.

Preimplantation development of fused bovine embryos

The cleavage rate of fused embryos was checked 3 days after electrofusion, and fused embryos were cultured until day 8 of embryonic development (day 0; day of electrofusion) in a medium (IVC-II) of the same composition of IVC-I, except that the BSA was replaced with 10% (v/v) FBS. The blastocyst rate was analyzed at day 8 after electrofusion. Eight times were replicated for detecting the optimal electrofusion protocol.

Karyotyping of hypothetical tetraploid blastocysts

Karyotyping was performed as previously described by Ulloa Ulloa et al. [28] with minor modifications. Blastocyst stage embryos were cultured in 0.5% (v/v) colcemid solution (Biological Industries Ltd., Kibbutz Beit Haemek, 25115, Israel) for 4–6 h in a humidified atmosphere of 5% CO_2 in air. Blastocyst embryos were rinsed in 0.9% (w/v) sodium citrate solution and kept at 37°C for 20 min. Embryos were mounted onto slides and fixed with a pre-fixative solution (methanol: acetic acid: distilled water=5:1:4), followed by a fixative solution (methanol: acetic acid=3:1). Slides were blocked in fixative solution at 4°C for overnight. Thereafter, embryos were stained with 4% (w/v) Giemsa staining solution for 5 min at room temperature. Slides were then rinsed thoroughly with distilled water. The karyotypes of all stained embryos were evaluated under a phase contrast microscope (Zeiss Axioskop, Germany).

DAPI staining procedures

After washing thrice with Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate buffered saline containing 0.1% (v/v) polyvinyl alcohol (PBS-PVA; Sigma-Aldrich), 2-cell stage and fused 2-cell stage embryos were fixed for 30 min in PBS-PVA containing 4% (w/v) paraformaldehyde. Fixed embryos were washed thrice with PBS-PVA and incubated overnight in 4°C in PBS supplemented with 2% (w/v) BSA (PBS-BSA) and 0.1%

(v/v) Triton X-100 (Nacalai Tesque Inc., Kyoto, Japan). After three washes in PBS-BSA for 10 min each, DNA was stained with 2 mg/ml DAPI (4', 6-diamidino-2-phenylindole dihydrochloride; Molecular Probes, Eugene, Oregon, USA) for 60 min. After washing the samples thoroughly, they were mounted onto slides and examined with an epifluorescence microscope (Olympus IX71, Tokyo, Japan).

Experimental design

The number of 2-cell stage and 3/4-cell stage embryos was counted every 2 h from 22 h to 38 h after IVF. Accordingly, embryos were classified as early (from 26 to 30 h after IVF) or late cleaved (from 30 to 36 h after IVF) to achieve developmental synchronization of 2-cell stage embryos for electrofusion. Only good quality 2-cell embryos were counted and collected for electrofusion. A single pulse of 0.75 V/cm (DC) for 60 µs and two pulses of 1.0 kV/cm (DC) for 25 µs were applied to identify the optimal electrofusion parameters. Unfused embryos were removed 2 h after electrofusion and used for fusion rate analysis. Cleavage rate and blastocyst rate of fused embryos were detected on days 3 and 8 after electrofusion, respectively. Karyotypes of day 8 blastocysts were determined by karyotype analysis procedures. Fused embryos were fixed at 0, 2, 4, 6, 8, 10, 12, 14 and 16 h after electrofusion and DNA was stained with DAPI to observe daughter nuclei development. To obtain a higher rate of tetraploid blastocyst formation, 3/4-cell stage embryos formed 10-12 h post electrofusion were classified as the hypothetical diploid group. The embryos that became 3/4-cell stage were classified as the hypothetical tetraploid group. Both groups were subsequently cultured to analyze the development rate after electrofusion.

Statistical analysis

Results were expressed as number and percentage. Karyotype rates of hypothetical tetraploid and diploid groups in day 8 blastocyst were analyzed by one-way ANOVA. Significant differences between groups were detected using Duncan's multiple range test. Differences with $P<0.05$ were considered significant. All statistical tests were performed with SPSS ver. 18 (SPSS Inc., Chicago, IL, USA).

Results

Analysis of early embryonic cleavage kinetics during first (2-cell Stage) and second (4-cell Stage) post-fertilization cell cycles

A total of 1,611 COCs were collected for IVM. Presumed zygotes (n=1,483) were selected for IVC1 culture, and cleavage time during first (2-cell) and second (4-cell) cell cycles was recorded. Development rate to 2- and 4-cell stage embryos was calculated from total presumed zygote numbers. Two-cell stage embryos were first detected (Figure 1) at 22 h after IVF (0.3%), and the transition from 1-cell to 2-cell was at the highest speed (18.9%) during 28-30 h after IVF. A few embryos completed the second cell cycle and cleaved into the 4-cell stage (0.3%) at 28 h post-insemination. Overall, 69.3% of embryos completed the first cell cycle from 22 to 38 h after IVF. To our knowledge, this is the largest numbers of bovine COCs and zygotes used for such investigation, therefore proving the best representation of the *in vitro* development kinetics of bovine IVF embryos.

Selection of good quality 2-cell embryos for electrofusion from the early (from 26 to 30 h after IVF) and late (from 30 to 36 h after IVF) cleavage groups

A total of 3,171 COCs were collected for IVM and used for IVF. Presumed zygotes (n=2,995) of good quality (see Materials and methods for the criteria used to select good quality embryos) were selected for
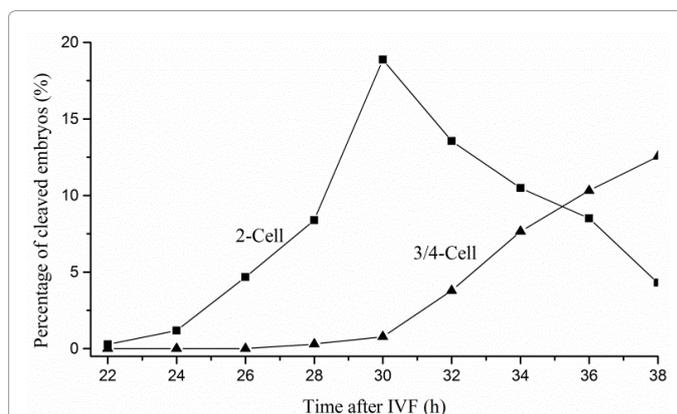

Figure 1: Early embryonic cleavage speed during second (2-cell stage) and third (4-cell stage) post-fertilization cell cycle.

Embryo cleavage time during the second and third post-fertilization cell cycle was detected every 2 h from 22 to 38 h after IVF. Early (from 26 to 30 h after IVF) and late (from and 30 h to 36 h after IVF) cleavage groups were divided to ensure developmental synchronization and sufficient numbers of good quality 2-cell stage embryos for electrofusion.

IVM	IVC-1	No. and (%) of embryos developed to at				
		26 h	26-30 h		30-36 h	
		Cleaved	Cleaved*	Good**	Cleaved	Good
3171	2995	254 (8.5)	770 (25.7)	517 (17.3)	838 (28.0)	369 (12.3)

*The cleaved embryos at the corresponding time after IVF

**The good quality 2-cell stage embryos in the cleaved ones. The cleaved and good quality 2-cell stage embryo rates were calculated from presumed zygote number

Table 1: Selection of good quality 2-cell stage embryos for electrofusion from the early (from 26 to 30 h after IVF) and late (from 30 to 36 h after IVF) cleavage group.

IVC1 culture and subsequent electrofusion when they reached 2-cell stage. Embryos cleaved before 26 h (8.5%) after IVF were not used for electrofusion as very low number of 2-cell stage embryos could be harvested. The proportion of early (from 26 to 30 h after IVF) and late (from 30 to 36 h after IVF) cleavage embryos was 25.7% and 28.0%, respectively. The percentage of good quality 2-cell stage embryos that were used for electrofusion was 17.3% at 30 h and 12.3% at 36 h (Table 1).

Effect of different electrofusion protocols on preimplantation development of fused embryos

We first investigated whether the number of electric pulses affects the fusion rate. As shown in Table 2, fusion rates were not significantly different in embryos obtained between using a single pulse of 0.75 kV/cm (DC) for 60 µs and using two pulses of 1.0 kV/cm (DC) for 25 µs. However, the fused embryos induced by a single pulse of 0.75 kV/cm (DC) for 60 µs had significantly higher blastocyst rates both in the early (45.4% *vs.* 24.4%) and late (30.9% *vs.* 15.2%) cleavage groups than their counterparts fused by two pulses of 1.0 kV/cm (DC) for 25 µs. The cleavage rate of the late (from 30 to 36 h after IVF) cleavage embryo group fused with 1.0 kV/cm (DC) was significantly lower compared to other experimental groups. Interestingly, the blastocyst rates of the early groups were all significantly higher ($P<0.05$) than that of the late groups regardless the electrofusion parameters.

Analysis of ploidy transition by monitoring nuclear configuration in electrofused 2-cell stage embryos

Nuclear configuration in electrofused 2-cell stage embryos derived from early cleavage group was monitored at 0, 2, 4, 6, 8, 10, 12, 14 and 16

Conditions of electric stimulation			Defined time quantum (h)	No. of 2-cell embryos collected from defined time quantum	No. and (%) of embryos developed to[*]		
DC (kV/cm)	Duration (μs)	Pulse times (n)			Fused	Cleavage	BL (D8[**])
0.75	60	1	Early (24-30)	298	251 (84.2)[a]	203 (80.9)[a]	114 (45.4)[a]
		1	Late (30-36)	243	207 (85.2)[a]	152 (73.4)[b]	64 (30.9)[b]
1.0	25	2	Early (24-30)	267	225 (84.3)[a]	200 (88.9)[a]	55 (24.4)[c]
		2	Late (30-36)	196	164 (83.7)[a]	92 (56.1)[c]	25 (15.2)[d]

[*]The fused rates were calculated from the No. of 2-cell embryos collected from defined time quantum, the cleavage and blastocyst rates were calculated from the fused embryo number

[**]Day 8 blastocysts (day 0; day of electrofusion)

[a-d]Values with different superscripts within same column are significantly different (*P*<0.05)

Table 2: Effect of different electrofusion protocols on preimplantation development of fused embryos.
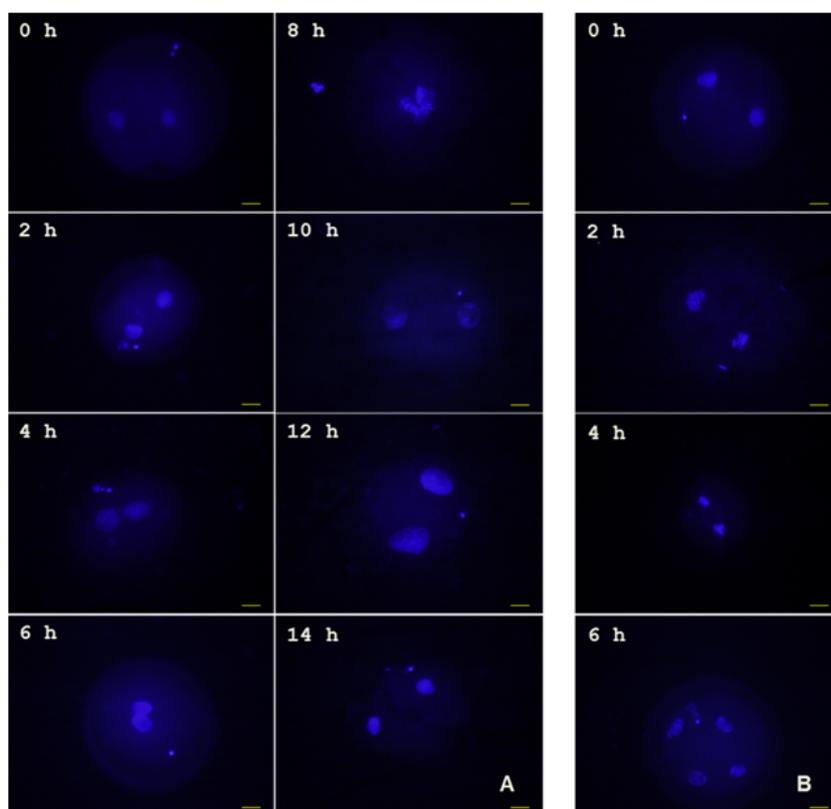

Figure 2: Visualization of nuclear development in bovine tetraploid (A) and diploid (B) embryos derived from electrofused embryos.

Fused embryos were fixed at 0, 2, 4, 6, 8, 10, 12, 14 and 16 h after electrofusion and DNA was stained with DAPI to observe nuclear reprogramming after electrofusion. **A)** Two nuclei from each embryo blastomere were tended to get close to each other and finally fused during 2 to 6 h post fusion. Fused embryos were at metaphase after 8 h of electrofusion, and completed the first cell cycle then came to 2-cell stage tetraploid embryos around 14 h after electrofusion. **B)** Two nuclei of fused diploid 2-cell stage embryos were detected at metaphase 4 h after electrofusion. Fluorescence visualization of one cell contained four nuclei was monitored at 6 h after electrofusion. Finally, the fused 2-cell stage embryos came to the 4-cell stage directly during 6-8 h after electrofusion. Scale bar in 100 μm of all pictures.

h after electrofusion by fluorescence staining (Figure 2). Two different types of nuclear configuration were detected when the embryos were examined at each of the time points post fusion. At 2 to 4 h post fusion, the first type of nuclear configuration is manifested as the two nuclei at interphase positioned close to each other while the second type as two separated metaphase nuclei positioned relatively far from each other; at 6 h post fusion, the first type as fully fused nuclei (25.8%) and the second type as 4 unfused nuclei (32.3%); at 8 h post fusion, the first type as fused metaphase tetraploid nuclei (30.0%) and the second type as diploid 4-cell stage embryos (30.0%); at 10-12 h post fusion, the first type finished tetraploid metaphase, and the second type were mostly in diploid 4-cell stage (50.0%); at 14 h post fusion, the first type finished the cell cycle and mostly in tetraploid 2-cell stage

embryos (23.3%) while the second type were diploid at 4-cell or more advanced stages. Even though the cell fixation procedures prevented us from monitoring the development of a fused embryo at all of the time points post fusion, based on the distinct cell cycle stages and nuclei positions between these two types of nuclear configurations, we extrapolated that the type-one nuclear configurations observed at each of the time points recapitulate the sequential nuclear changes of what a tetraploid embryo would undergo; similarly, the type-two nuclear configurations are unique to the diploid embryos. As shown by the karyotyping analysis of the two groups of blastocysts classified based on the different timings of completing 4-cell cleavage cell cycle post fusion, from fused 2-cell embryos (see below), such reasoning seemed to be substantiated.

Assessment of preimplantation development in hypothetical tetraploid and diploid embryos

As mentioned above, 2-cell stage embryos collected from early (from 26 to 30 h after IVF) and late (from 30 to 36 h after IVF) cleavage groups were used for electrofusion. Our initial observation on the cleavage of the fused 2-cell embryos indicated that some of them became 4-cell stage embryos 10-12 h after electrofusion while other became 4-cell stage embryos only after this post electrofusion time point (Table 3). Based on the two types of nuclear configurations observed above, we hypothesized that the 4-cell stage embryos formed 10-12 h after electrofusion were still diploid while the ones taking more than 12 h to become 4-cell stage embryos were tetraploid. Consequently, we classified the 4-cell stage embryos developed 10–12 h after electrofusion as the hypothetical diploid group, whereas the remaining embryos as the hypothetical tetraploid group. Our hypothesis was corroborated by the karyotyping studies on blastocysts developed from these embryos (see below). We found that the percentages of hypothetical diploid 4-cell stage embryos are very similar between the early (48.2%) and the late (51.8%) cleavage groups, indicating that the kinetics of the first cleavage of fertilized embryos (to form 2-cell stage diploid embryos) does not dictate the kinetics of the first cleavage of fused 2-cell stage embryos (to form either hypothetical tetraploid or 4-cell stage embryos).

Karyotype analysis of blastocyst derived from hypothetical tetraploid group and hypothetical diploid group

A total of 132 blastocysts from fused 2-cell stage embryos of hypothetical tetraploid group (n=59) and hypothetical diploid group (n=73) were used for karyotype analysis. Consistent with our hypothesis, significantly higher percentage of blastocysts were identified as tetraploid in the hypothetical tetraploid group than in the hypothetical diploid group, regardless they are from the early cleavage group or the late cleavage group (51.5% *vs.* 7.5% for early cleavage group; 53.8% *vs.* 6.1% for late cleavage group) (Table 4). Therefore,
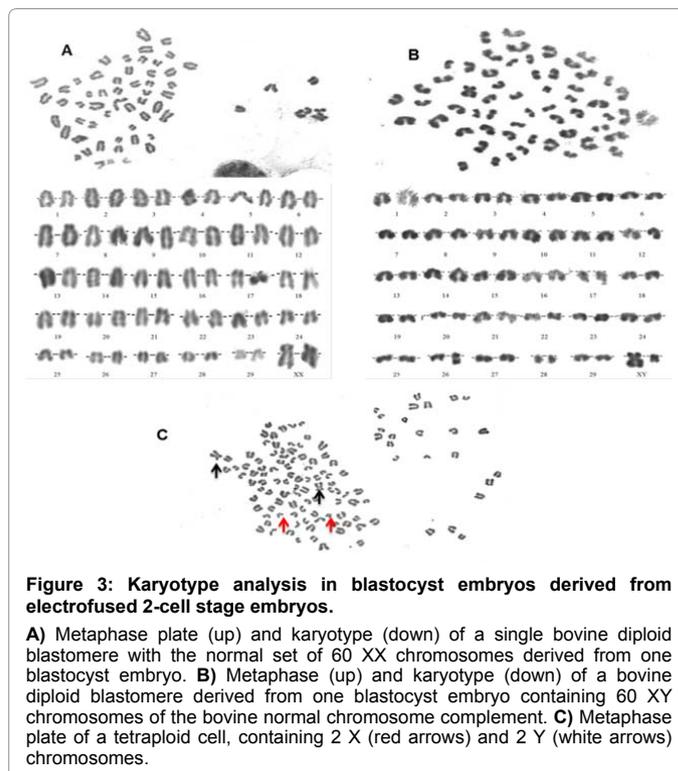

Figure 3: Karyotype analysis in blastocyst embryos derived from electrofused 2-cell stage embryos.

A) Metaphase plate (up) and karyotype (down) of a single bovine diploid blastomere with the normal set of 60 XX chromosomes derived from one blastocyst embryo. **B)** Metaphase (up) and karyotype (down) of a bovine diploid blastomere derived from one blastocyst embryo containing 60 XY chromosomes of the bovine normal chromosome complement. **C)** Metaphase plate of a tetraploid cell, containing 2 X (red arrows) and 2 Y (white arrows) chromosomes.

we have established a strategy to efficiently produce tetraploid bovine blastocysts by simply selecting the hypothetical tetraploid 2-cell stage embryos based on the kinetics of the first cleavage of the fused 2-cell embryos; with this protocol, up to 53.8% of the blastocyst are tetraploid (Table 4) (Figure 3).

Discussion

The mouse tetraploid embryo system has been successfully established by electrofusing 2-cell stage embryos [22,29-32]. Mouse 2-cell stage embryos are produced *in vivo* by mating superovulated females primed with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). Since ovaries and follicles are homochronous, mouse embryos develop into the 2-cell stage at almost the same time. In addition, mouse embryos require at least 20 h to develop from the 2-cell to the 3/4-cell stage [33]. In bovine, ovaries obtained from an abattoir for *in vitro* embryo production are variable in estrous cycle stage and follicles size. This heterogeneity of the follicular populations results in retrieval of COCs with different developmental potentials that vary in cleavage timing during the first (2-cell stage) and second (4-cell stage) post-fertilization cell cycles [34,35]. Furthermore, bovine embryos require 8-10 h to complete the second cell cycle and to reach the 4-cell stage [36,37], indicating that bovine embryos finish the second cell cycle (4-cell stage) more quickly than mouse embryos. Therefore, compared to mouse, it is much more challenging to obtain synchronous 2-cell stage bovine embryos either *in vivo* or *in vitro*.

Previous studies have described electrofusion of 2-cell stage bovine embryos selected at 28 to 30 h [1] and 33 to 35 h [23,24,38] post IVF. In the present study, we analyzed the timing of the first and second cell cycles of bovine embryos. In our experiments, 2-cell stage embryos were first detected at 22 h, reached to the peak of 2-cell formation at 30 h, then declined sharply to reach lower detectable number at 36 h post insemination. Based on this, embryos were classified into either early (from 26 to 30 h after IVF) and late (from and 30 h to 36 h after IVF)

Cleavage group	No. and (%) of embryos developed into			
	2-cell stage	Fused	Separation of tetraploid and diploid	BL (D8)
Early	443	384 (86.7)[a]	HDip[*]	185 (48.2)[a]	108 (58.4)[b]
			HTetra[**]	199 (51.8)[a]	56 (28.1)[a]
Late	359	307 (85.5)[a]	HDip	159 (51.8)[a]	66 (41.5)[c]
			HTetra	148 (48.2)[a]	30 (20.3)[a]

[*]Hypothetical diploid group, the 4-cell stage embryos were selected during 10–12 h after electrofusion

[**]Hypothetical tetraploid group, the remaining embryos were selected after picking out the hypothetical diploid embryos. The HDip and HTetra rates were calculated from the number of fused one

[a-c]Values with different superscripts within same column are significantly different (*P*<0.05)

Table 3: Assessment of preimplantation development of hypothetical tetraploid and diploid embryos in early and late cleavage groups.

Cleavage group	Hypothetical tetraploid and diploid group	Tetraploid (%)	Diploid (%)	Mixoploid (%)
Early	HDip	3 (7.5)[b]	28 (70.0)[b]	9 (22.5)[a]
	HTetra	17 (51.5)[a]	13 (39.4)[a]	3 (9.1)[a]
Late	HDip	2 (6.1)[b,c]	26 (78.8)[c]	5 (15.2)[a]
	HTetra	14 (53.8)[a]	8 (30.8)[a]	4 (15.4)[a]
Total		36 (27.8)	75 (56.8)	21 (15.9)

[a-c]Values with different superscripts within same column are significantly different (*P*<0.05)

Table 4: Analysis of karyotype rates of hypothetical tetraploid and diploid groups in day 8 blastocyst.

cleavage group to ensure developmental synchronization and sufficient numbers of good quality 2-cell stage embryos for electrofusion. The percentage of cleaved embryos obtained as early (25.7%) was nearly the same as the late cleaved group (28.0%). However, the percentage of good quality 2-cell stage embryos from early cleaved (17.3%) was higher than that of late cleaved ones (12.3%).

The relationship between fusion rate and different electrofusion parameters (e.g., number of electric pulses, direct current [DC] strength and DC durations), in addition to the mechanism of cell membrane electrofusion, were described in somatic cells [39]. For fusion 2-cell stage mammalian embryos, different electrofusion parameters were used [23,40] according to the type of fusion buffer, electrofusion instrument and animal species [38]. In mice, the highest tetraploid blastocyst formation rate (93.0%) of fused embryos was achieved when electrofusion was performed using 20-volt AC and 100-volt DC [41]. In rabbits, tetraploid embryos were obtained with a single pulse of 1.4 kV/cm for 100 μs and 74.5% of them developed to morula and blastocyst stages, with 72.7% of the morulae/blastocysts being tetraploid [40]. In cattle, on the other hand, even though comparable fusion rates can be achieved for 2-cell stage bovine embryos [23,24,38], the great majority of the blastocysts developed from the fused embryos are diploid [24]. In the present study, we used one pulse of 0.75 kV/cm for 60 μs and two pulses of 1.0 kV/cm for 25 μs for bovine 2-cell stage embryos electrofusion. Our data showed that one pulse of 0.75 kV/cm for 60 μs resulted in the highest rate (45.4%) of blastocyst formation when early two-cell stage embryos were used for electrofusion compared to late counterparts (30.9%). The cleavage timing of embryos used for electrofusion was an additional variable that we considered to determine the most suitable condition for producing tetraploid bovine embryos. The percentage of viable blastocyst embryos (15.2%) decreased greatly when late cleaved embryos were used or the voltage was increased to two pulses of 1.0 kV/cm for 25 μs.

Electrofusion of 2-cell stage embryos results in both tetraploid and diploid embryos, but the nuclear configuration and the kinetics of nuclear fusion after electrofusion of 2-cell stage bovine embryos had not been studied before. Therefore, in the present study, nuclear configuration and the kinetics of nuclear fusion in cytoplasmic membrane-fused 2-cell stage bovine embryos produced by our optimized protocol were investigated. Our objectives were not only to shed lights on the process of nuclear fusion but also to test the possibility of whether tetraploid embryos can be separated from diploid embryos by simply basing on the timing of the fused embryos to become 4-cell stage embryos again. Fluorescence visualization of fused 2-cell stage embryos showed that the tetraploid and diploid embryos undergo different timing to finish the first cell cycle to form 2-cell stage embryos. In tetraploid embryos, the nuclei of fused 2-cell stage embryos underwent chromosomal replication, nuclear membrane fusion, followed by accomplishment of the first cell cycle to become 2-cell stage tetraploid embryos at 14 h post of electrofusion. On the other hand, for the reasons yet to be discovered, the nuclei in a subset of the fused embryos never fused together and came to 4-cell stage diploid embryos directly as early as 6-8 h after fusion. Four-cell stage embryos formed within 12 h post electrofusion were classified as the hypothetical diploid group and were removed from fused embryos. Our karyotyping analysis at the blastocyst stage demonstrated that significantly higher tetraploid rates were observed in the hypothetical tetraploid group than in the hypothetical diploid group of both early (51.5% *vs.* 7.5%) and late (53.8% *vs.* 6.1%) cleavage groups (Table 4). This tetraploid rate of blastocysts is more than 4-fold higher than what was achieved by the previous research [24]. We also detected 9.1-22.5% of blastocyst as mixoploidy, which is a common phenomenon in domestic animal species as shown in earlier studies [42,43].

We reasoned that early stage 2-cell embryos are more probable to produce tetraploid after electrofusion as these fused 2-cell stage embryo nuclei are at more synchronized in cell cycle and at earlier stage that would allow them to have adequate time to undergo nuclear membrane fusion and chromosomal replication before they reach to the metaphase. However, very comparable tetraploid rate were found between the fused early and late cleavage 2-cell stage embryos (53.8% *vs.* 51.5%). Very significantly, our study detected the distinct nuclear remodeling events between tetraploid and diploid embryos post 2-cell fusion in bovine by showing that the bovine tetraploid and diploid embryos go through different cell cycles. Based on such novel finding, we developed an easy and quick method to effectively separate the diploid and tetraploid embryos according to their different cell cycle time intervals. In conclusion, we achieved a high tetraploid rate (53.8%) in bovine and believe that such protocol can be widely adopted by different laboratories interested in using tetraploid bovine embryos for transgenic research and for the study of bovine embryo development.

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References

1. Iwasaki S, Campbell KH, Galli C, Akiyama K (2000) Production of live calves derived from embryonic stem-like cells aggregated with tetraploid embryos. Biol Reprod 62: 470-475.

2. Heyman Y (2005) Nuclear transfer: a new tool for reproductive biotechnology in cattle. Reprod Nutr Dev 45: 353-361.

3. Beyhan Z, Forsberg EJ, Eilertsen KJ, Kent-First M, First NL (2007) Gene expression in bovine nuclear transfer embryos in relation to donor cell efficiency in producing live offspring. Mol Reprod Dev 74: 18-27.

4. Hochedlinger K, Jaenisch R (2002) Monoclonal mice generated by nuclear transfer from mature B and T donor cells. Nature 415: 1035-1038.

5. Eggan K, Baldwin K, Tackett M, Osborne J, Gogos J, et al. (2004) Mice cloned from olfactory sensory neurons. Nature 428: 44-49.

6. Li J, Ishii T, Feinstein P, Mombaerts P (2004) Odorant receptor gene choice is reset by nuclear transfer from mouse olfactory sensory neurons. Nature 428: 393-399.

7. Wang Z, Jaenisch R (2004) At most three ES cells contribute to the somatic lineages of chimeric mice and of mice produced by ES-tetraploid complementation. Dev Biol 275: 192-201.

8. Wakayama S, Jakt ML, Suzuki M, Araki R, Hikichi T, et al. (2006) Equivalency of nuclear transfer-derived embryonic stem cells to those derived from fertilized mouse blastocysts. Stem Cells 24: 2023-2033.

9. Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, et al. (2007) Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nat Genet 39: 295-302.

10. Eakin GS, Hadjantonakis AK, Papaioannou VE, Behringer RR (2005) Developmental potential and behavior of tetraploid cells in the mouse embryo. Dev Biol 288: 150-159.

11. Jouneau A, Zhou Q, Camus A, Brochard V, Maulny L, et al. (2006) Developmental abnormalities of NT mouse embryos appear early after implantation. Development 133: 1597-1607.

12. Zhao XY, Lv Z, Li W, Zeng F, Zhou Q (2010) Production of mice using iPS cells and tetraploid complementation. Nat Protoc 5: 963-971.

13. Kang L, Gao S (2012) Pluripotency of induced pluripotent stem cells. J Anim Sci Biotechnol 3: 5.

14. Eakin GS, Behringer RR (2003) Tetraploid development in the mouse. Dev Dyn 228: 751-766.

15. Modlinski JA (1978) Transfer of embryonic nuclei to fertilised mouse eggs and development of tetraploid blastocysts. Nature 273: 466-467.

16. Sembon S, Fuchimoto D, Iwamoto M, Suzuki S, Yoshioka K, et al. (2011) A simple method for producing tetraploid porcine parthenogenetic embryos. Theriogenology 76: 598-606.

17. Bai C, Liu H, Liu Y, Wu X, Cheng L, et al. (2011) Diploid oocyte formation and tetraploid embryo development induced by cytochalasin B in bovine. Cell Reprogram 13: 37-45.

18. Eglitis MA (1980) Formation of tetraploid mouse blastocysts following blastomere fusion with polyethylene glycol. J Exp Zool 213: 309-313.

19. Krivokharchenko A, Galat V, Ganten D, Bader M (2002) *In vitro* formation of tetraploid rat blastocysts after fusion of two-cell embryos. Mol Reprod Dev 61: 460-465.

20. O'Neill GT, Speirs S, Kaufman MH (1990) Sex-chromosome constitution of post-implantation tetraploid mouse embryos. Cytogenet Cell Genet 53: 191-195.

21. Kubiak JZ, Tarkowski AK (1985) Electrofusion of mouse blastomeres. Exp Cell Res 157: 561-566.

22. Clement-Sengewald A, Brem G (1989) Electrofusion parameters for mouse two-cell embryos. Theriogenology 32: 159-169.

23. Iwasaki S, Kono T, Fukatsu H, Nakahara T (1989) Production of bovine tetraploid embryos by electrofusion and their developmental capability *in vitro*. Gamete Res 24: 261-267.

24. Curnow EC, Gunn IM, Trounson AO (2000) Electrofusion of two-cell bovine embryos for the production of tetraploid blastocysts *in vitro*. Mol Reprod Dev 56: 372-377.

25. Deb GK, Dey SR, Bang JI, Cho SJ, Park HC, et al. (2011) 9-cis retinoic acid improves developmental competence and embryo quality during in vitro maturation of bovine oocytes through the inhibition of oocyte tumor necrosis factor-Î± gene expression. J Anim Sci 89: 2759-2767.

26. Jeong WJ, Cho SJ, Lee HS, Deb GK, Lee YS, et al. (2009) Effect of cytoplasmic lipid content on *in vitro* developmental efficiency of bovine IVP embryos. Theriogenology 72: 584-589.

27. Wright RW, Ellington J (1995) Morphological and physiological differences between *in vivo*- and *in vitro*-produced pre-implantation embryos from livestock species. Theriogenology 44: 1167-1189.

28. Ulloa Ulloa CM, Yoshizawa M, Yamashita A, Hama S, Mitsui A, et al. (2008) Blastocyst production from in vitro-produced day-2 bovine embryos classified by cleavage stage, and cytogenetical evaluation of the resultant day-8 blastocysts. J Reprod Dev 54: 465-472.

29. Eakin GS, Hadjantonakis AK (2006) Production of chimeras by aggregation of embryonic stem cells with diploid or tetraploid mouse embryos. Nat Protoc 1: 1145-1153.

30. McLaughlin KJ (1993) Production of tetraploid embryos by electrofusion. Methods Enzymol 225: 919-930.

31. Petzoldt U (1995) Electrofusion of mouse embryos to produce tetraploids. Methods Mol Biol 48: 331-340.

32. Kubiak JZ, Tarkowski AK (1985) Electrofusion of mouse blastomeres. Exp Cell Res 157: 561-566.

33. Sawicki W, Abramczuk J, Blaton O (1978) DNA synthesis in the second and third cell cycles of mouse preimplantation development. A cytophotometric study. Exp Cell Res 112: 199-205.

34. Barnes FL, Eyestone WH (1990) Early cleavage and the maternal zygotic transition in bovine embryos. Theriogenology 33: 141-152.

35. Hay-Schmidt A, Viuff D, Greve T, Hyttel P (2001) Transcriptional activity in *in vivo* developed early cleavage stage bovine embryos. Theriogenology 56: 167-176.

36. Holm P, Shukri NN, Vajta G, Booth P, Bendixen C, et al. (1998) Developmental kinetics of the first cell cycles of bovine *in vitro* produced embryos in relation to their *in vitro* viability and sex. Theriogenology 50: 1285-1299.

37. Lechniak D, Pers-Kamczyc E, Pawlak P (2008) Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos. Reprod Biol 8: 23-42.

38. Mohamad RD, Mohamad HN, Hosein B, Mohmad M, Hojatolah K (2008) Fusion and development of 2-cell bovine embryos to tetraploid blastocyst with different voltages anddurations. Iranian J Reprod Med 6: 181-186.

39. Abidor IG, Sowers AE (1992) Kinetics and mechanism of cell membrane electrofusion. Biophys J 61: 1557-1569.

40. Liu L, An M (1993) [Electrofusion of two-cell embryos of rabbit and their developmental capacity *in vitro*]. Yi Chuan Xue Bao 20: 493-498.

41. Park MR, Lee AR, Bui HT, Park C, Park KK, et al. (2011) Chromosome remodeling and differentiation of tetraploid embryos during pre-implantation development. Dev Dyn 240: 1660-1669.

42. Viuff D, Rickords L, Offenberg H, Hyttel P, Avery B, et al. (1999) A high proportion of bovine blastocysts produced *in vitro* are mixoploid. Biol Reprod 60: 1273-1278.

43. Booth PJ, Viuff D, Tan S, Holm P, Greve T, et al. (2003) Numerical chromosome errors in day 7 somatic nuclear transfer bovine blastocysts. Biol Reprod 68: 922-928.