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Production of blastocysts from aggregates of buffalo embryonic stem cells and putative tetraploid buffalo embryos

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ABSTRACT

This study was aimed at producing tetraploid (4*n*) buffalo embryos and their aggregates with buffalo embryonic stem (ES) cells. In experiment 1, immature oocytes obtained from slaughterhouse buffalo ovaries were subjected to IVM and IVF after which the blastomeres of 2-cell stage embryos were electrofused using 4 combinations of pulse field strength (0.75 or 1.25 kV/cm) and pulse duration (50 or 99 µsec). Although the differences in fusion (72–82%), cleavage (76–83%) and blastocyst rate (5–16%) among the 4 groups were not significant, the highest blastocyst rate was obtained with 1.25 kV/cm and 99 µsec. Chromosome analysis of electrofused embryos revealed that among the 30 spreads examined, 5 (16.6%) were tetraploid whereas the rest 83.3% were not tetraploid. In experiment 2, following culture of aggregates of buffalo ES cells stained with Hoechst 33342 and putative tetraploid embryos at 8- to 16-cell stage, 1/47 (2.1%) aggregates prepared from ES cells and IVF-derived 8- to 16-cell embryos developed to blastocyst stage whereas when 56 aggregates prepared from ES cells and parthenogenesis-derived 8- to 16-cell stage embryos were cultured, 16 (28.5%) developed to blastocyst stage. These results demonstrated that tetraploid buffalo embryos can be produced by electrofusion and that these embryos can be aggregated with buffalo ES cells to produce blastocysts. Aggregates prepared from parthenogenetically-derived embryos have better developmental competence than those prepared from IVF-derived embryos.

Key words: Aggregation, Buffalo, Electrofusion, Embryonic stem cells, Tetraploid embryos

The tetraploid embryos, having double complement of chromosomes (4*n*) instead of the usual diploid one (2*n*), are a useful model for generating valuable information on embryonic development. Their use has enabled the development of an experimental system in which the carrier blastomeres were tetraploid rather than diploid (Tarkowski *et al.* 2001, 2005). Fusion of 2 diploid blastomeres of the 2-cell stage embryos is the most commonly used method for production of tetraploid embryos in mice (Kaufman and Webb 1990), rabbit (Ozil and Modlinski 1986), pig (Prather *et al.*1996) and cattle (Iwasaki *et al.* 1999, Curnow *et al.* 2000).

Tetraploid embryos can develop to the blastocyst stage but do not lead to birth of an offspring. Homogeneously tetraploid (4*n*) mouse embryos reportedly developed to mid-gestation before spontaneously aborting since polyploidy is generally incompatible with the normal development of most mammalian tissues, except the extra-embryonic tissues which naturally possess subpopulations of polyploid cells (James *et al.* 1995). Tetraploid embryos were used for generating mice directly from embryonic stem (ES) cells by tetraploid embryo complementation (Nagy *et al.* 1993, Wang *et al.* 1997, Eakin and Hadjantonakis 2006). The resulting chimeric preimplantation embryos can develop to term after transfer to a surrogate mother. Using 4*n* complementation, ES↔4*n* mice were derived from ES cells from embryos produced by IVF (Poueymirou *et al.* 2007) or somatic cell nuclear transfer (SCNT, Amano *et al.* 2009) and from induced pluripotent stem (iPS) cells (Kang *et al.* 2009, Zhao *et al.* 2009). In cattle, live chimeric calves were produced from aggregates derived from tetraploid embryos and inner cell mass (ICM) cells (Picard *et al.* 1990) or ES cell-like cells (Iwasaki *et al.* 2000) showing that ICM or ES cells could contribute to chimera formation.

We have developed buffalo ES cell lines that have been maintained for over 150 passages in our laboratory (Sharma *et al.* 2011). Live buffalo offspring were also successfully produced by SCNT using somatic (Shah *et al.* 2009) or ES

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cell (George *et al*. 2010) as the nucleus donor in our laboratory. However, the possibility of producing cloned calves by aggregation of buffalo ES cells with buffalo tetraploid embryos has not been explored. The ability to form a live offspring through this approach is regarded as the most stringent characterization test for the pluripotency of ES cells. This technique offers the advantage of being able to produce animals that could be classified as true clones rather than genomic copies as produced by SCNT since in this case the mitochondria would also be derived from the same cell as the nuclear genetic material. There is no report on the production of tetraploid embryos or their aggregates with ES cells in buffalo. The present study was, therefore, carried out to determine parameters for the production of tetraploid buffalo embryos and to explore the possibility of producing blastocysts following their aggregation with buffalo ES cells.

MATERIALS AND METHODS

Production of 2-cell embryos: Buffalo ovaries collected from a nearby abattoir were washed 3 times with warm isotonic saline (32–37°C) containing 400 I.U/ml penicillin and 500 mg/ml streptomycin and were transported to the laboratory within 6 h. Follicular (2 to 8 mm in diameter) oocytes were aspirated with a 19 gauge needle attached to a 10 ml syringe in the aspiration medium (TCM 199 + 0.3% BSA + 50 mg/ml gentamicin sulphate). The oocytes were then washed 4–6 times with the washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 50 mg/ml gentamicin sulphate). Only those cumulus-oocytes complexes (COCs) which had a compact and unexpanded cumulus mass having ≥2 layers of cumulus cells, and with homogenous evenly granular ooplasm were used for *in vitro* maturation (IVM). After washing 3 times with IVM medium (TCM 199 + 10% FBS + 10% buffalo follicular fluid + 1 µg/ml estradiol-17ß + 5 mg/ml pFSH + 0.81 mM sodium pyruvate + 0.68 mM glutamine + 50 mg/ml gentamicin sulphate), groups of 15–20 COCs were cultured in 100 µl droplets of the IVM medium, overlaid with sterile mineral oil in 35 mm Petri dishes, and cultured for 24 h in a humidified CO_2 incubator (5% CO_2 in air) at 38.5ºC.

For production of 2-cell stage embryos by *in vitro* fertilization (IVF), the spermatozoa were processed as described earlier (Chauhan *et al*. 1998) with some modifications. Briefly, 2 straws of frozen thawed buffalo semen were washed twice with the washing Bracket and Oliphant (BO) medium (BO medium containing 10 µg/ml heparin, 137.0 µg/ml sodium pyruvate and 1.942 mg/ml caffeine sodium benzoate). The pellet was resuspended in around 0.5 ml of the capacitation and fertilization BO medium (washing BO medium containing 10 mg/ml fatty acid-free BSA). The *in vitro* matured oocytes were washed twice with the fertilization BO medium and transferred to 50 µl droplets (15–20 oocytes/droplet) of the capacitation and fertilization BO medium. The spermatozoa in 50 µl of the capacitation and fertilization BO medium (2–4 million spermatozoa/ml) were then added to the droplets containing the oocytes, covered with sterile mineral oil and placed in a CO_2 incubator (5% CO_2 in air) at 38.5°C for 18 h for IVF. At the end of sperm-oocyte incubation, the cumulus cells were washed off the oocytes by gentle pipetting. The oocytes were then washed several times with modified Charles Rosenkrans medium with amino acids (mCR2aa) containing 0.6% BSA and cultured in this medium for 33–35 h on original beds of cumulus cells.

For production of 2-cell stage embryos by parthenogenesis, *in vitro* matured oocytes were activated (~24 h after the start of maturation) by incubation in T20 (where T denotes HEPES modified M-199 supplemented with 2.0 mM L-glutamine, 0.2 mM sodium pyruvate, 50 µg/ml gentamicin sulphate and the following number denotes FBS in percent v/v) containing 5 µM calcimycin A23187 for 5 min at 38.5ºC. The oocytes were washed 3 times with T20 and were then incubated in medium containing 2 mM 6-dimethylaminopurine (6-DMAP), covered with mineral oil and incubated in a CO_2 incubator at 38.5ºC for 4 h. The activated oocytes were washed several times with RVCL containing 1% fatty acid-free BSA and cultured in this medium for 33–35 h on original beds of cumulus cells.

Production of tetraploid embryos: Embryos at the 2-cell stage were briefly exposed (<5 min) to fusion buffer (0.3 M D-mannitol + 0.1 mM $MgSO_4$ + 0.05 mM $CaCl_2$ + 1 mg/ml polyvinyl alcohol) for pre-equilibration and were then transferred to the fusion chamber. The embryos were placed between the electrodes of microslide fusion chamber filled with fusion buffer that was connected to a BTX electrocell manipulator. After exposure to the electric pulse, the embryos were washed with T20 and transferred to droplets of IVC medium (mCR2aa + 0.6% BSA + 10% FBS) on original beds of granulosa cells. Embryos in which the blastomeres were found to be fused within 1 h of exposure to electric current were cultured further for 7 days post-insemination to examine their developmental competence, using 2-cell embryos which had not been subjected to electrofusion as controls. Cleavage was assessed 12 h post fusion and blastocyst development was recorded on day 7 post fusion. Alternatively, the fused embryos were cultured till they reached the 8- to 16-cell stage at which time they were used for forming aggregates with buffalo ES cells.

Chromosome analysis of embryos: Blastomeres of diploid or putative tetraploid 4-cell stage embryos were arrested at the metaphase stage by culturing the embryos in the presence of 100 µg/ml colchicine for 18 h after which these were treated with 0.9% tri-sodium citrate for 10–15 min and were then transferred to clean grease-free glass slides. Immediately later, the embryos were burst with a fine pointed glass needle, and approximately 2 µl of fixative-1 (methanol: acetic acid in a 1: 1 ratio) was dropped on the embryo. Just before the first droplet of fixative dried, a second droplet of fixative-2

(methanol: acetic acid in a 3: 1 ratio) was added after which the slides were air dried for 18–24 h and stained with 10% Giemsa stain for 30 min. The chromosome preparations were observed at 1000× under oil immersion.

Processing of buffalo ES cells: Buffalo ES cells (passage 17–19) that had been produced in the laboratory and had been characterized (George *et al.* 2010, Sharma *et al.* 2011) were used in the present study for forming ES cell-putative tetraploid aggregates.

For thawing of cryopreserved ES cells, the straws were plunged into a water-bath at 37°C for 30 sec. The sealed ends were cut and the ES cell colonies were transferred to the holding medium (HM) that consisted of DMEM + 20% FBS + 0.2 M sucrose for 1 min of incubation. After this, the colonies were incubated for 5 min in HM containing 0.1 M sucrose. The colonies were further incubated twice (5 min each) in HM without sucrose before being seeded on mitomycin C (10 µg/ml)-treated buffalo fetal fibroblast feeder layer, which was prepared as described by Sharma *et al.* (2011). The ES cells were cultured in ES cell medium (Knockout DMEM + 15% Knockout serum replacement + 1000 I.U./ml mLIF + 5 ng/ml bFGF + 2 mM L-glutamine + 50 µg/ml gentamicin sulphate + 1% MEM non-essential amino acids + 0.1 mM ß-mercaptoethanol) and the colonies were mechanically passaged every 5–6 days in a split ratio of 1: 2 or 1: 3 depending upon their size. Immediately prior to culture of ES cell-putative tetraploid embryo aggregates, colonies of ES cells were mechanically removed from the feeder layer with a microblade and were treated with trypsin-EDTA for 2–3 min to remove any residual cells of the feeder layer. The colonies were dissected into small clumps and stained with Hoechst 33342 (10 µg/ml for 10 min).

Production of ES cell-tetraploid embryo aggregates: Well-of-the-wells (WOWs), prepared a day prior to aggregation with the help of darning needles in wells of 4-well dishes (15–20 microwells per well), were washed 5–6 times with DPBS containing 50 µg/ml gentamicin sulphate after which 400 µl FBS was dispensed in each well and the dish was incubated at 38.5°C in a CO_2 incubator for 12 h. After this, FBS was replaced with an equal volume of RVCL(R) (Research vitro cleave) medium supplemented with 1% fatty acid-free BSA and was covered with sterile mineral oil. The zona pellucida was removed from putative tetraploid 8- to 16-cell stage embryos with 0.5% pronase after which the embryos were washed with T20 medium. Two zona-free putative tetraploid embryos and one clump of ES cells stained with Hoechst 33342 were transferred to the WOW in such a manner that the clump of ES cells was placed in the depression, sandwiched between the 2 tetraploid embryos. The aggregates were cultured at 38.5°C in a humidified CO_2 incubator (5% CO_2 in air) for the next 6 days.

Experimental design and statistical analysis: In experiment 1, the effect of pulse duration and pulse field strength on the fusion efficiency and developmental competence of fused putative tetraploid embryos was examined, combinations of 2 different pulse field strengths (0.75 and 1.25 kV/cm) and pulse durations (50 and 99 µsec) were compared. Embryos that had not been subjected to electrofusion were used as controls. In experiment 2, the developmental competence of aggregates of ES cell-putative tetraploid 8- to 16-cell stage embryos was compared for embryos derived either from IVF or from parthenogenesis. The data were analyzed using SYSTAT 6.0 after arcsine transformation of percentage values. The differences between means were analyzed by one-way ANOVA followed by Fisher's LSD test. Significance was determined at $P<0.05$.

RESULTS AND DISCUSSION

In experiment 1, it was found that the fusion efficiency and, cleavage and blastocyst rate were not significantly different for the pulse field strengths of 0.75 and 1.25 kV/cm, and pulse durations of 50 and 99 µsec (Table 1; Fig. 1). Chromosome analysis of electrofused 2-cell stage embryos revealed that 5/30 (16.6%) spreads were tetraploid whereas the rest 83.3% were not tetraploid, and among the non-electrofused controls, none of the spreads was found to be tetraploid (Table 2; Fig. 2). In experiment 2, when 47 aggregates prepared from ES-cells and IVF-derived 8- to 16-cell stage embryos were cultured, 1 (2.1%) developed to the blastocyst stage whereas when 56 aggregates prepared

Table 1. Developmental competence of buffalo embryos subjected to electrofusion

Electrofusion parameters		Treated 2-cell embryos (n)	Fused embryos	Development after fusion	
Pulse field strength (kV/cm)	Pulse duration (µsec)		n (%)	Cleaved n (%)	Blastocysts n (%)
1.25	50	58	47(80.9±7.3)[a]	38(79.5±4.9)[a]	2 (4.7±2.8)[a]
1.25	99	71	52(72.4±8.6)[a]	42(76.2±10.0)[a]	9(15.8±6.3)[a]
0.75	50	68	52 (75.8±5.3)[a]	43(82.6±3.1)[a]	2(4.8±3.0)[a]
0.75	99	66	54(81.8±1.8)[a]	41(76.1±6.2)[a]	5(11.4±4.6)[a]
Control (not subjected to electrofusion)		66	-	-	24(36.4±3.2)[b]

Data from 4 trials. Values with different superscripts within the same column differ significantly ($P<0.05$).

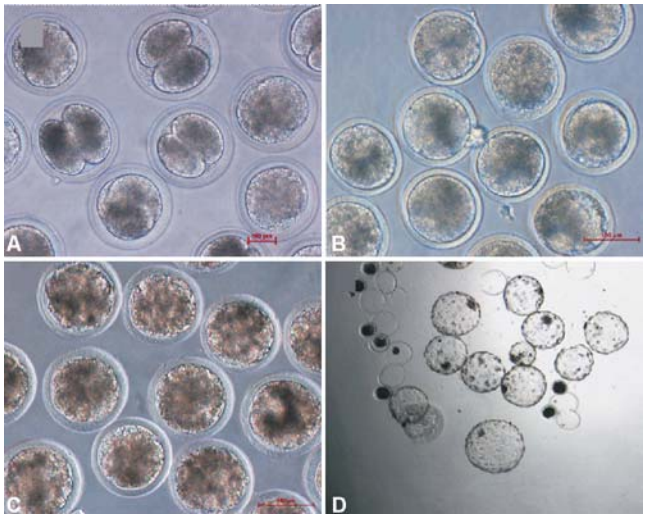

Fig. 1. *In vitro* produced buffalo embryos at: (A) 2-cell stage before electrofusion, (B) with fused blastomeres 2 h after electrofusion. Putative tetraploid embryos at: (C) 8- to 16-cell stage, and (D) blastocyst and hatched blastocyst stages.

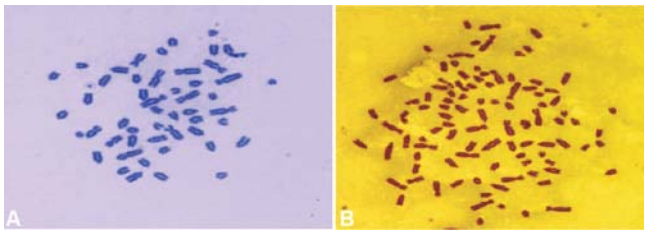

Fig. 2. Metaphase spread of IVF embryos showing: (A) normal 2n diploid chromosome complement, and (B) 4n tetraploid chromosome complement.

Table 2. Chromosome composition of cells obtained from electrofused embryos

Embryos	Number of spreads examined (n)	Non-tetraploid n (%)	Tetraploid -4n n (%)
Electrofused	30	25(83.3)	5(16.6)
Control	15	15(100)	0(0)

from ES-cells and parthenogenesis-derived 8- to 16-cell stage embryos were cultured, 16 (28.5%) developed to the blastocyst stage (Table 3; Fig. 3).

To our knowledge, this is the first report on the production of tetraploid embryos and ES cell-tetraploid embryo aggregates in buffalo. When combinations of 2 pulse field strengths i.e. 0.75 kV/cm and 1.25 kV/cm and 2 pulse durations 50 µsec and 99 µsec were compared, although the differences in fusion, cleavage and blastocyst rate among the 4 groups were not statistically significant, the highest blastocyst rate was obtained with 1.25 kV/cm and 99 µsec suggesting that this combination can be used for efficient electrofusion of buffalo embryos. Also, there was a clear trend

Table 3. Developmental capability of putative tetraploid embryo-ES cell aggregates.

Method of producing putative tetraploid embryos	Number of aggregates cultured (n)	Number of blastocysts formed n (%)
IVF	47	1 (2.1)
Parthenogenesis	56	16 (28.5)

IVF data from 5 trials. Parthenogenesis data from 3 trials.

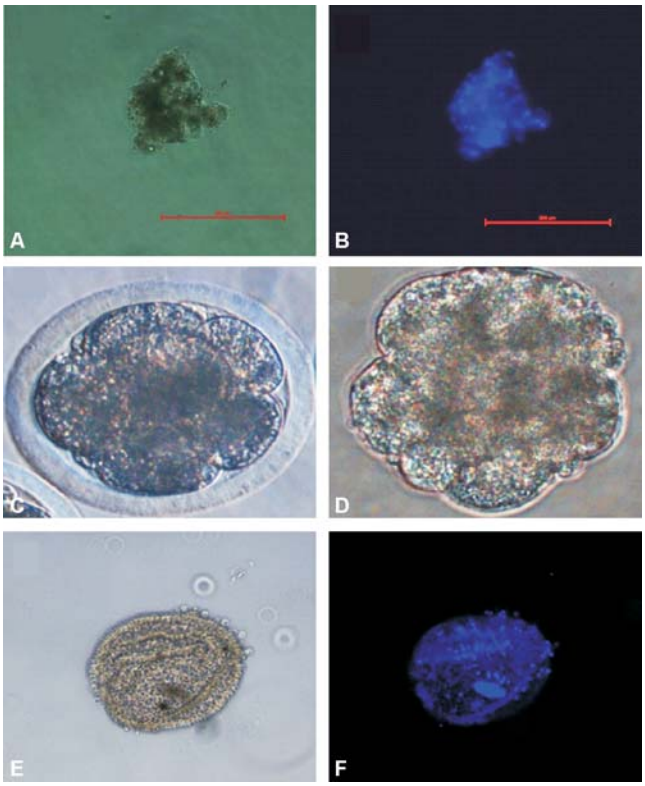

Fig. 3. (A) Clump of ES cells to be used for preparing ES cell-tetraploid embryo aggregate, under bright light, and (B) under fluorescence, after being stained with Hoechst 33342, (C) a putative tetraploid embryo at 8- to 16-cell stage, (D) after removal of zona pellucida, (E) a blastocyst developed from ES cell-putative tetraploid embryos aggregate under bright field, and (F) under fluorescence.

that the blastocyst rate was lower with 50 µsec compared to that with the higher pulse duration of 99 µsec irrespective of the pulse field strength. The fusion, cleavage and blastocyst rate in the present study is similar to that obtained following exposure of 2-cell stage cattle embryos to 2 pulses of 1.0 kV/cm each of 25 µsec duration (Iwasaki *et al.* 1999, 2000) or 1.4 kV/cm as a single 100 µsec pulse (Curnow *et al.* 2000).

Electrofusion leads to dissolution of the cell membrane between the two blastomeres resulting in production of a single cell with two 2*n* sets of chromosomes. When 4-cell stage embryos formed from electrofused embryos were

subjected to chromosome analysis, 16.6% spreads were found to be tetraploid, which is similar to that of 12.5% obtained following electrofusion of cattle IVF-derived embryos (Curnow *et al.* 2000). However, others have found nearly 80% of electrofused cattle embryos to be tetraploid (Iwasaki *et al.* 1999). The reason for this is not clear. Nevertheless, these figures are higher than those of non-electrofused embryos among which none was found to be tetraploid. These results highlight the need to confirm the tetraploidy before aggregation, especially in view of the possibility of diploid cells in the embryos overcoming tetraploid cells (Iwasaki *et al*. 2000), which may lead to production of chimeric offspring.

Following the culture of ES cell-putative tetraploid embryo aggregates, only 2.1% developed to the blastocyst stage when IVF-derived embryos were used. This is much lower than the blastocyst rate of 16–37% reported in cattle (Iwasaki *et al.* 1999, 2000). It is difficult to pinpoint the reason for this since a large number of factors such as species, stage of tetraploid embryo, nature and passage number of ES cells, positional relationship between ES cells and tetraploid embryos, *in vitro* culture conditions employed etc. influence development of the aggregates. Tetraploid embryos at the completely compacted morula stage were found to be less efficient in forming aggregates with ES cells compared to partially compacted or compacting embryos in cattle (Iwasaki *et al.* 1999). We have, therefore, used 8- to 16-cell stage embryos, as also used in earlier studies in cattle (Iwasaki *et al.* 2000). Viable mice that were entirely ES cell-derived were produced following aggregation of ES cells and tetraploid embryos at the 4-cell stage (Nagy *et al.* 1993). The aggregation rate was reported to be poor if a clump of ICM cells was sandwiched between 2 clumps of putative tetraploid embryos on a flat surface compared to when the clump was put in the depression made by 'darning needle' to form a triangle with putative tetraploid embryos in cattle (Iwasaki *et al.* 1999). We have, therefore, used the latter technique in our study. This technique is simple and does not require a high level of skill or expensive equipment. The number of passages also affects the developmental pluripotency of ES cells. In one study, from the mice produced by tetraploid embryo complementation with ES cells, among all the mice that reached adulthood and that could reproduce, none arose from ES cells at passage level 15 or more, all arose from cells at passages 3–11 (Li *et al.* 2007). The optimal passage number of ES cells for cattle or buffalo is not known. Since the proportion of aggregates that develop to the blastocyst stage is very low in all the reports in cattle and in the present study in buffalo, each of the factors mentioned above warrants further investigation.

The blastocyst rate was much higher (28.5%) following aggregation of ES cells with putative tetraploid embryos derived by parthenogenesis. To our knowledge, this is the first report on the production of aggregates from ES cells and tetraploid embryos produced by parthenogenesis. It needs to be investigated whether parthenogenetically-derived tetraploid embryos can contribute to the extra-embryonic tissues as effectively as those produced by IVF.

Buffalo ES cells stained with Hoechst 33342 were used for the aggregation to identify the cells of blastocysts that the ES cells contributed to. It was found that in all the blastocysts formed from the aggregates, the ICM cells exhibited fluorescence indicating that the ICMs were derived mainly if not exclusively from the ES cells. In a majority of blastocysts, some cells from trophectoderm were also found to exhibit fluorescence suggesting that trophectoderm was derived partly from putative tetraploid embryos. This is consistent with previous studies on cell distribution in $4n \leftrightarrow 2n$ chimeras in which the tetraploid cells made a significantly greater contribution to the trophectoderm, specifically the mural trophectoderm, compared to the ICM (James *et al.* 1995).

Transgenic chimeric cattle were produced using bovine embryo-derived ES-like cells at passage 10 in which the ES-like cells contributed to gonadal tissue of the offspring (Cibelli *et al*. 1998). Production of blastocysts from aggregates of buffalo ES cells and tetraploid buffalo embryos in the present study needs to be used further for paving the way for production of transgenic buffaloes by aggregating transgenic ES cells with tetraploid embryos.

In conclusion, it was demonstrated in the present study that tetraploid buffalo embryos can be produced by electrofusion and that these embryos can be aggregated with buffalo ES cells to produce blastocysts. Aggregates prepared from parthenogenetically-derived embryos have better developmental competence than those prepared from IVF-derived embryos.

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