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—Original Article—

Effects of Repetitive Ionomycin Treatment on *In Vitro* Development of Bovine Somatic Cell Nuclear Transfer Embryos

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Abstract. To artificially activate embryos in somatic cell nuclear transfer (SCNT), chemical treatment with ionomycin has been used to induce transient levels of Ca^{2+} and initiate reprogramming of embryos. Ca^{2+} oscillation occurs naturally several times after fertilization (several times with 15- to 30-min intervals). This indicates how essential additional Ca^{2+} influx is for successful reprogramming of embryos. Hence, in this report, the experimental design was aimed at improving the developmental efficiency of cloned embryos by repetitive Ca^{2+} transients rather than the commonly used ionomycin treatment (4 min). To determine optimal Ca^{2+} inflow conditions, we performed three different repetitive ionomycin (10 μM) treatments in reconstructed embryos: Group 1 (4-min ionomycin treatment, once), Group 2 (30-sec treatment, 4 times, 15-min intervals) and Group 3 (1-min treatment, 4 times, 15-min intervals). Pronuclear formation rates were checked to assess the effects of repetitive ionomycin treatment on reprogramming of cloned embryos. Cleavage rates were investigated on day 2, and the formation rates of blastocysts (BLs) were examined on day 7 to demonstrate the positive effect of repeated ionomycin treatment. In Group 3, a significant increase in BL formation was observed [47/200 (23.50%), 44/197 (22.33%) and 69/195 (35.38%) in Groups 1, 2 and 3, respectively]. Culturing embryos with different ionomycin treatments caused no significant difference among the groups in terms of the total cell number of BLs (164.3, 158.5 and 145.1, respectively). Additionally, expression of the anti-apoptotic *Bcl-2* gene and *MnSOD* increased significantly in Group 3, whereas the expression of the pro-apoptotic *Bax* decreased statistically. In conclusion, the present study demonstrated that repeated ionomycin treatment is an improved activation method that can increase the developmental competence of SCNT embryos by decreasing the incidence of apoptosis.
Key words: Apoptosis, Bovine embryos, Ca^{2+} oscillation, SCNT

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Ca^{2+} oscillation is a universal signaling cue that activates numerous cellular responses. Ca^{2+} signaling is ubiquitous in some somatic cells and germ lines. In particular, in early embryo development, the fluctuating Ca^{2+} concentration is a factor that brings about serial developmental mechanisms [1]. After fertilization, matured oocytes arrested in the metaphase stage of the second meiotic division (MII) turn into zygotes with subsequent signaling events such as depolarization, Ca^{2+} oscillation, cortical reaction and pronucleus formation [2, 3]. The exit from MII resumption and the further processes for forthcoming development do not occur before the concentration of intracellular Ca^{2+} is increased by sperm penetration. Sperm is the initial signal for Ca^{2+} release mediated by IP_3 (inositol trisphosphate), protein tyrosine kinases and PLC gamma (PLC-ζ in mammals), followed by reorganization of Ca^{2+}-releasing machinery to trigger subsequent stages [4, 5]. Up until the two pronuclei merge together, repetitive Ca^{2+} influx (called Ca^{2+} oscillation) occurs repeatedly [5].

The mechanism of Ca^{2+} change in embryos varies widely among species. Zygotes of lower vertebrates have a single and long Ca^{2+} release cue, while mammalian eggs repetitively emit Ca^{2+} over a short term of several hours, referred to as Ca^{2+} oscillation [6–9]. In mammals, the initial activation of oocytes is induced by intracellular Ca^{2+} and supported by the influx of extracellular Ca^{2+}. The fluctuation of the Ca^{2+} concentration in the cytoplasm of embryos tends to occur repeatedly within several minutes after activation. It appears that these transient Ca^{2+} peaks respectively are involved in different cellular events. It has been shown that although a single activation with an electric pulse or chemical treatment of parthenogenetic embryos might initiate further development, repeated Ca^{2+} signals could provide more complete coordination of several events, such as cortical granule formation, cell cycle and protein synthesis [10, 11]. Some reports on animal embryos demonstrated that the Ca^{2+} induction number, frequency, amplitude and duration are vital factors that regulate many individual cellular events, emphasizing the importance of Ca^{2+} release and influx in the early development of embryos [12, 13].

Thus, optimizing the activation method for enucleated oocytes is a critical step in cloned animal production. Because Ca^{2+} influx alone is not enough to activate embryos, sequential combination or

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single treatment with chemicals such as ionomycin, Ca-ionophore/6-DMAP and artificial electrical stimuli have been used as typical ways to activate nuclear transferred (NT) embryos [14, 15], while cycloheximide and cytochalasin B have been used to activate NT embryos of certain species [16–18]. In the same way, many trials of new combinations of chemicals and adjustments of the activation time have been performed in various species [2, 19–21].

We considered that repeated ionomycin treatment, to induce extracellular Ca^{2+} inflow and release of cytosolic Ca^{2+} stores, would improve the production rate of cloned embryos. According to a recent report, animal oocytes activated in medium with high $CaCl_2$ showed a higher developmental rate than that of their counterpart, supporting the importance of additional Ca^{2+} inflow [2]. However, no reported study has used repeated ionomycin treatments to induce several Ca^{2+} surges in bovines. To follow the natural mechanism of the Ca^{2+} oscillation occurring at intervals of a few minutes, NT oocytes were repeatedly treated with ionomycin in this experiment. Additionally, expression of developmentally important genes and apoptosis-related genes were analyzed for the effects of the repeated ionomycin treatment using quantitative real-time PCR.

Materials and Methods

In vitro maturation

Bovine ovaries were obtained from a local slaughterhouse. Cumulus-oocyte complexes (COCs) were retrieved from antral follicles, 3–8 mm in diameter, by aspiration with an 18-gauge needle attached to a 10 ml syringe. The COCs with homogenous ooplasm and that were enclosed by more than three layers of compact cumulus cells were selected. For maturation, a group of 30–40 COCs were cultured for 22 h in bicarbonate-buffered TCM-199 supplement with 10% (v/v) FBS, 0.005 IU/ml follicle-stimulating hormone, 1 mg/ml sodium pyruvate, 1 μg/ml 17 β-estradiol and 0.001 IU/ml luteinizing hormone at 38 C in a humidified atmosphere of 5% CO_2.

Preparation of donor cells

Fibroblast cells were isolated from the ears of Holstein heifers and washed several times with phosphate-buffered saline (PBS) plus a 5% antibiotic solution. The washed tissues were minced into several small pieces with a sharp blade in DMEM containing 10% FBS and then cultured in 100 mm^2 culture plates in a humidified, 5% CO_2 incubator at 38 C. After five days, the cultured fibroblasts that were more than 90% confluent were trypsinized and plated in new dishes.

Somatic cell nuclear transfer

After 22 h of maturation culture, expanded cumulus cells of the COCs were removed by repeated pipetting in 0.1% (v/v) hyaluronidase. Oocytes with a first polar body and homogenous ooplasm were enucleated with a micromanipulator. Bovine skin fibroblast cells from a calf were transferred into the perivitelline space of the enucleated oocytes, and then these NT couplets were treated with electrical fusion (2 pulses, 1.75 kV cm^{-1}, 15 μsec). Reconstructed embryos were subsequently cultured in SOF medium (5% CO_2, 5% O_2 and 38 C).

Activation

At 2 h after electrical fusion, the fused embryos were randomly divided into three groups. The fused oocytes of each group were differentially treated with 5 μM ionomycin [Group 1 (4-min ionomycin treatment, once), Group 2 (30-sec treatment, 4 times, 15-min intervals) and Group 3 (1-min treatment, 4 times, 15-min intervals)]. After the first ionomycin treatment, reconstructed embryos were incubated for 4 h in SOF supplemented with 1.9 mM 6-dimethylaminopurine (6-DMAP) under a humidified atmosphere with 5% CO_2 at 38 C.

In vitro development and counting cell numbers of blastocysts

After 6-DMAP treatment, the reconstructed embryos were cultured in 25 μl microdrops of SOF under mineral oil in groups of 3–10 embryos for 168 h. Embryo development to the two-cell (cleavage) and blastocyst (BL) stages was evaluated at 48 h and 168 h of culture, respectively. In order to count total cell numbers of blastocysts, after washing three times in washing media (0.5% BSA/PBS), blastocysts were stained with 0.01% (w/v) bisbenzimide for 2 min. Stained blastocysts were mounted with glycerol (Sigma-Aldrich) on glass slides under a coverslip and examined under an inverted microscope (Nikon, Tokyo, Japan) with a counter.

Quantitative gene expression analysis

Quantitative gene expression analysis was conducted using real-time PCR. Three BLs from each replicate were used for analyzing the expression levels. The same experiment was repeated at least four times for each group. Messenger RNAs were selectively isolated from each replicate using a Dynabeads mRNA Direct kit, following the manufacturer's instructions. Extracted mRNAs were mixed with a High Capacity RNA-to-cDNA Kit (Applied Biosystems, cat. 4387406) and incubated at 37 C for 1 h. Complementary DNAs were conserved in a final volume of 20 μl elution buffer to give a final PCR reaction volume of 20 μl, including 1 μl of template. Each sample was analyzed with *beta-actin* as an internal control. Detection was performed with an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using a quantitative real-time PCR kit (Applied Biosystems) and SYBR green (Power SYBR Green PCR Master Mix, Applied Biosystems) under the following PCR reaction conditions: 95 C for 15 min and 40 cycles of denaturation at 95 C for 15 sec, annealing at 60 C for 30 sec and extension at 72C for 30 sec. The sequences of primers used and accession numbers are summarized in Table 1. The relative expression ratios and threshold cycle (Ct) values of genes normalized by beta-actin (dCt) were calculated using the 2Δ Ct method.

Statistical analysis

Differences in embryo development rate and in gene expression assessed by quantitative RT-PCR among these experimental groups were then analyzed using a one-way ANOVA test. Tukey's test was then used to determine significant differences among experimental groups. Significant differences among the treatments were determined when the P-value was < 0.05.

Table 1. Primer sequences and real-time PCR conditions

Gene name		Sequence	bp	Accession number
Oct-4	F	ACACTCGGACCACGTCTTTC	110	NM_174580
	R	AGTGAGAGGCAACCTGGAGA		
Nanog	F	CCAGCAGATGCAAGAACTTTCC	158	DQ 069776
	R	GAAGCCTGGGTATTCTGCCATT		
Bax	F	CGCATCGGAGATGAATTGGA	167	NM_173894
	R	TAGAAAAGGGCGACAACCCG		
Bcl-2	F	CGCATCGTGGCCTTCTTTGAGTT	141	XM_586976
	R	GCCGGTTCAGGTACTCAGTCAT		
Hsp70	F	CGTGGCTCCCCTGTCGCT	140	U09861
	R	GCTGGTTGTCCGAGTAGGTGGT		
MnSOD	F	CTGGGGATTGACGTGTGGGA	132	L22092
	R	GCTGCAAGCCGTGTATCGTG		
IFN	F	CCTGGGAAATCATCAGAATGGAG	135	X65539
	R	TGTGGCATCTTAGTCAGCGAGAG		
Cx43	F	TGGAGAGGGAGGGGATGAGG	135	NM174068
	R	GGGGTGTGTGGGAAAGAAAAAG		
beta-actin	F	ATCACCATCGGCAATGAGCG	135	NM_173979
	R	GCACCGTGTTGGCGTAGAGG		

Results

In vitro development rates of reconstructed embryos

To assess the effect of repeated transient ionomycin treatment for 1 h on cloned embryos, an experiment was designed using two different experimental groups and a control group: Group 1 (4-min ionomycin treatment, once), Group 2 (30-sec treatment, 4 times, 15-min intervals) and Group 3 (1-min treatment, 4 times, 15-min intervals). In total, 5 μM ionomycin was used to treat the embryos for 4 min after fusion, and the samples were then incubated in 1.9 mM of DMAP drops for 4 h. This is a well-known routine protocol for bovine *in vitro* cloned and parthenogenetic embryos. According to a report, the patterns of Ca^{2+} oscillation in mammalian eggs persist for several hours after sperm fusion, occurring at regular 15- to 30-min intervals [5]. As such, the embryos in Group 2 were incubated in ionomycin drops for 30 sec and then washed in culture medium; the process was repeated another 3 times at 15-min intervals, and finally, the embryos were incubated in DMAP drops for 4 h. The same methods were used to treat the embryos in Group 3, though in this case, the embryos were incubated in ionomycin for 1 min before washing in culture medium in each repetition. Each experiment lasted 5 h, from the time of the first ionomycin treatment to the completion of DMAP incubation. For the control embryos, the start of DMAP incubation was delayed after ionomycin activation so that the timing of the experiment was synchronized with that of the other treatment groups.

The reconstructed embryos from each group were analyzed by checking pronuclear formation rates 18 h after activation (Table 2). There were considerably more embryos with normal pronuclei in the experimental groups, which underwent repeated ionomycine activation, than in the control group, which was treated using the conventional activation method (Group 1 (control), 35/65; Group 2, 44/65; and Group 3, 43/65; see Table 2). In the conventional activation group, there were more inactivated embryos with a condensed nucleus and abnormal embryos than in the repeated activation groups.

In order to demonstrate the effects of repeated ionomycin activation on embryo development during preimplantation, the number of embryos in each experimental group that entered a particular developmental phase was counted. Table 3 illustrates the significant differences in developmental rates between the experimental groups and the control group. The proportion of embryo cleavage in Group 2 was statistically higher than that in the control group (one-way ANOVA, $P<0.05$), but was not significantly different from that in Group 3. The morula formation rates showed a statistical difference in Group 3 (ionomycin, 1 min, 4 times) compared with those in Groups 1 and 2 (one-way ANOVA, $P<0.05$). Additionally, the BL formation rate of bovine embryos in Group 3 was significantly higher [69/200 (35.38%)] at 168 h postactivation compared with embryos in Group 1 (4 min, 1 time) and Group 2 (30 sec, 4 times) [47/200 (23.50%), 44/143 (22.33%), respectively]. However, the different ionomycin treatment method did not lead to significant differences among the groups in terms of the total cell numbers of BLs (164.3, 158.5 and 145.1, respectively). As can be seen in Figure 1, there was also no significant difference in terms of the quality of blastocysts among the groups (Fig. 1).

Expression of developmentally important genes

To determine the impact of repeated ionomycin treatment on the intracellular transcripts of embryos, we analyzed the expression of apoptotic-related genes and developmentally important genes in BLs from the three groups (Fig. 2). It was reported previously that the cell death pathway, which involves *Bax* and *Bcl-2*, is regulated by Ca^{2+} signaling during the development of preimplantation embryos; this was confirmed by detecting Ca^{2+}-dependent apoptotic mediators and measuring apoptotic gene expression [22–27]. Thus,

Table 2. Proportion of pronuclear formations of cloned bovine embryos with different repetitive ionomycin treatments

Group (Ionomycin treatment)	Number of			
	Total embryos	Embryos with normal pronucleus (%)	Embryos with condensed nucleus (%)	Abnormal embryos (%)
1 (4 min / 1 time)	65	35 (53.84) [a]	17 (26.15)	12 (18.46)
2 (30 sec / 4 times)	65	44 (67.69) [b]	11 (16.92)	7 (10.76)
3 (1 min / 4 times)	65	43 (66.15) [b]	9 (13.84)	9 (13.84)

[a, b] Superscripts in the same column represent significant differences between groups (P<0.05).

Table 3. Developmental competence of SCNT embryos activated with repetitive ionomycin treatments

Group (Ionomycin treatment)	Number of				No. of stained embryos	No. of total cells (mean±SEM)
	Embryos cultured	Embryos cleaved (%)	Morulae (%)	BLs (%)		
1 (4 min / 1 time)	200	121 (60.50)[a]	38 (19.00) [a]	47 (23.50) [a]	52	164.3± 20.7
2 (30 sec / 4 times)	197	143 (72.58)[b]	54 (27.41) [a]	44 (22.33) [a]	50	158.5 ± 32.2
3 (1 min / 4 times)	195	131 (67.17)[ab]	63 (32.30) [b]	69 (35.38)[b]	48	145.1 ± 17.8

[a, b] Superscripts in the same column represent significant differences between groups (P<0.05).

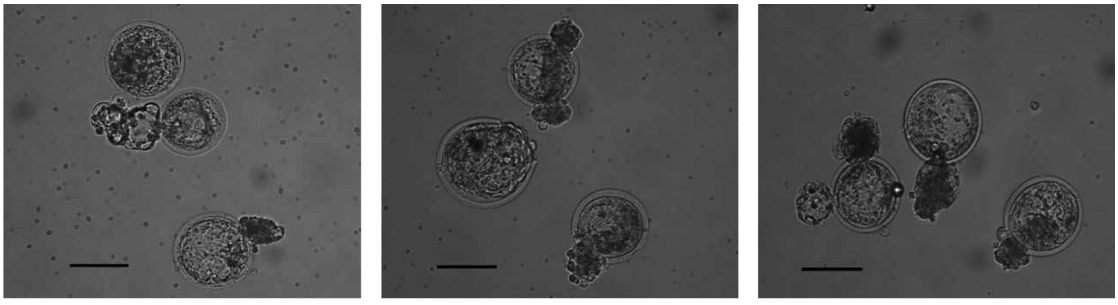

Fig. 1. Cloned bovine blastocysts of Group 1 (left), Group 2 (center) and Group 3 (right). Bar represents 100 μm.

we detected gene expression of *Bax* (an apoptosis indicator) and *Bcl-2* (an antiapoptotic regulator) in the BLs from the different experimental groups. *Bax* expression levels decreased slightly in Groups 2 and 3 compared with those in the control group, while the expression rate of *Bcl-2* increased significantly in Group 3 (Fig. 2B). In addition, we checked the expression of *Hsp70* (70 kDa heat shock protein), which plays a role in the protection of cells from stress, and manganese superoxide dismutase *(MnSOD*, which is important in antioxidant defense), in the three groups. There was no significant difference in the alteration of *Hsp70* expression, but the *MnSOD* expression level in Group 3 increased more than fourfold compared with that in the control group (Fig. 2B).

Oct4, Nanog (pluripotent-related genes), *Cx43* (gap junction protein) and *Interferon tau* (transcription factors in early trophoblast cells) gene were analyzed to examine the normality of the blastocysts in the experimental groups in terms of intracellular transcripts. *Cx43*, a maternal gene important in early embryo development, is a critical protein in preimplantation development. It is a marker of developmental potential for bovine embryos derived from oocytes with different developmental competences; in other words, low *Cx43* expression is an indicator of blastocysts with low developmental competence, which is commonly seen in thawed embryos [28, 29]. *Interferon tau* is a key factor that controls the expression of several trophoblast-related factors that prepare the uterus for placental attachment, alter the uterine immune system and regulate early conceptus development [30, 31]. Therefore, the expression of *Interferon tau* was analyzed in order to check whether there was improved development of trophoblast cells in any of the experimental groups. No significant differences in levels of *Oct4, Nanog* and *Interferon tau* were noted among the experimental groups. Although it was not significant, the expression level of *Cx43* was higher in Groups 2 and 3 than in Group 1, the control group in which the routine protocol was used (Fig. 2B).

Discussion

This comparative study of two groups that underwent several repeated treatments with ionomycine and one group that used the conventional method of ionomycin treatment focused on proving the benefits of mimicking repetitive calcium oscillation. The results of this study demonstrate that repeated ionomycin treatment benefits the development of *in vitro* embryos during preimplantation. The

repeated ionomycin activation treatment groups (Groups 2 and 3) showed higher developmental competence than the control group that underwent conventional treatment (see Tables 2 and 3). These observations may be explained as the positive consequences of the multiple ionomycin treatments. That is, repeated ionomycin treatment leads to a higher and longer Ca^{2+} inflow and release, enough to continue fluctuating until the resumption of meiotic and MPF inactivation [23–25]. Although the embryos in Group 2

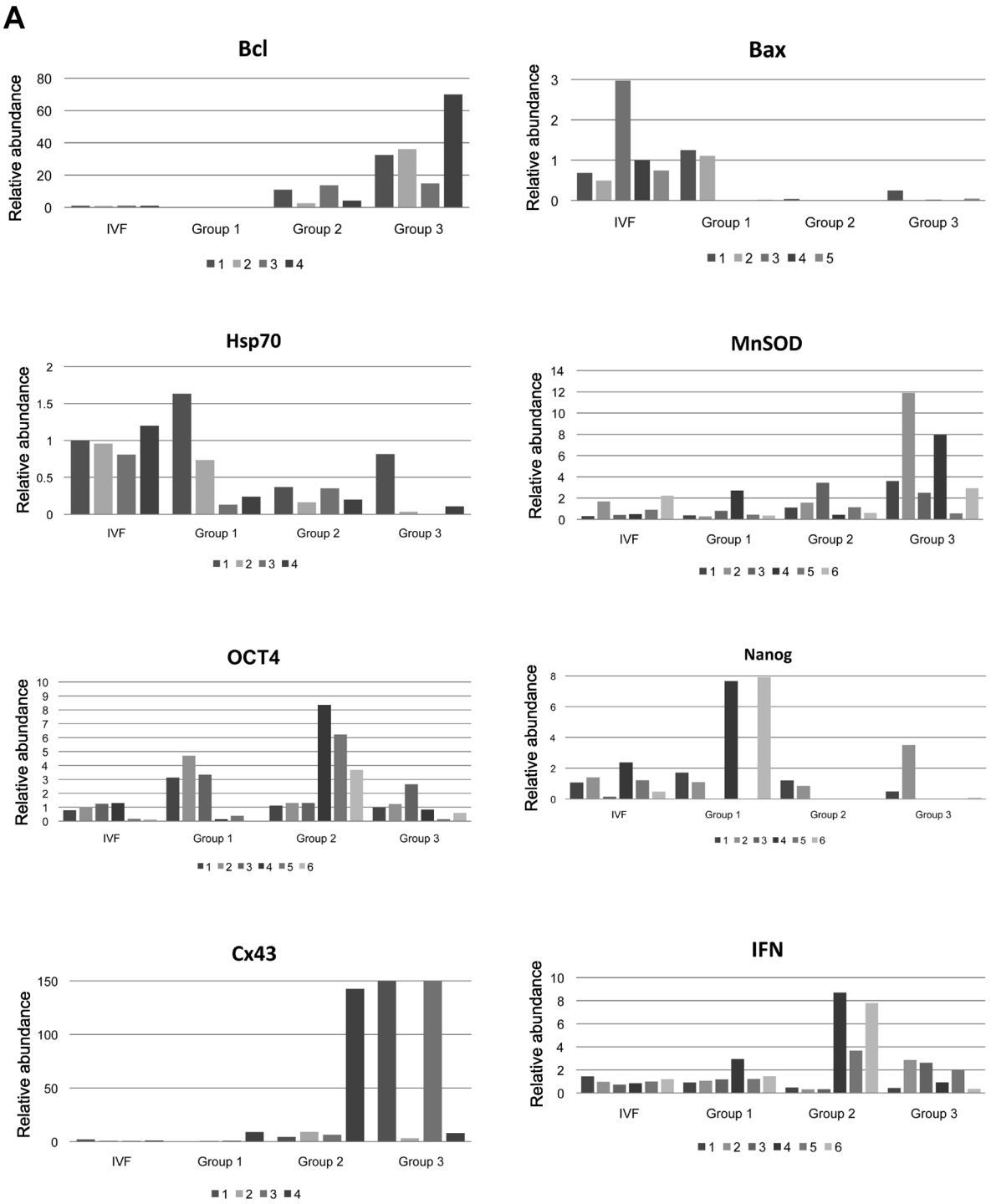

Fig. 2. Relative transcript levels of blastocysts in the three groups with different treatment systems as analyzed by real-time PCR. A: Relative abundance of various transcripts in each of 4-6 replicates from each treatment group. B: The means of four or six replicates were calculated for the control and experimental groups, and a standard error bar is shown for each group. Different superscripts represent significant differences ($P<0.05$).

could have been affected by a shortage of Ca^{2+} influx due to the short exposure time (30 sec), the BL formation rate in Group 2 was similar to that of the control group. This supports the idea that a repeated treatment system is a better activation method than longer exposure to ionomycin. The lower BL formation rate in Group 2 compared with that of group3, however, might have been caused by this shortage of Ca^{2+} influx compared with that of Group 3. This also demonstrates that a higher intracellular level of ionomycin is necessary to bring about improved subsequent development of activated oocytes, which is consistent with the findings of a previous report [2]. Considering the increased morula and BL formation rates in the repeated groups, it seems that the later Ca^{2+} inflow

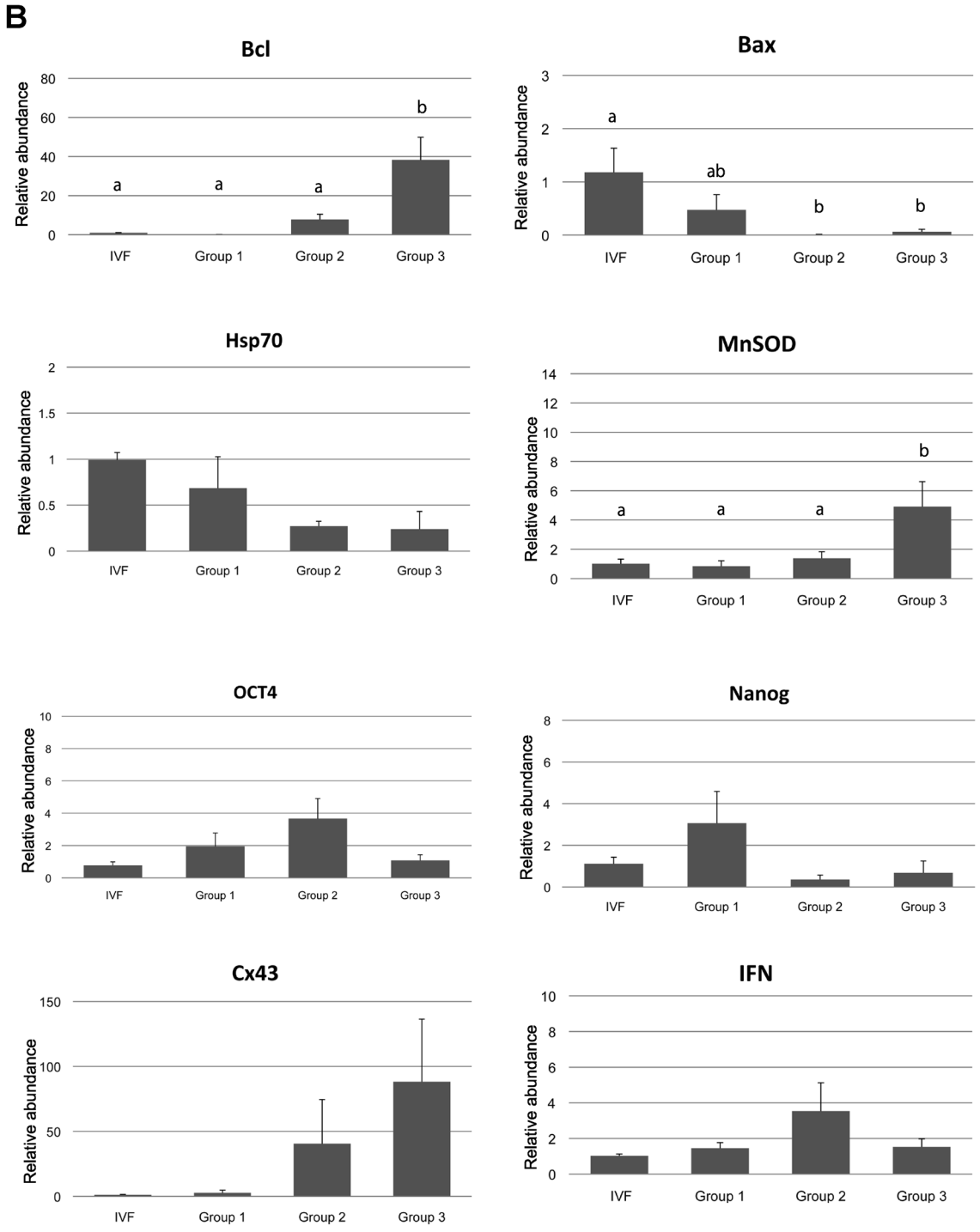

Fig. 2. Continued.

caused by additional ionomycin treatments influences not only early egg activation events, including the activities of MPF and MAPK, that lead to pronuclear formation, but also later embryonic development during preimplantation. This is consistent with results showing that higher and longer Ca^{2+} inflow caused by additional activation with ionomycin supports a better developmental rate in the BL stage [26, 27].

In addition, tests showing the gene expression levels of developmentally important genes confirmed the beneficial effects of repetitive ionomycin treatment on the development of *in vitro* bovine embryos during preimplantation. There was significant variation in the relative abundance of mRNA in each replicate as well as variation in the expression levels of certain genes (Fig. 2A). While there was no significant between group difference for some developmentally important genes (*Oct4, Nanog*, *Cx43, Interferon tau and Hsp70),* apoptosis-related genes *(Bax and Bcl)* and an antioxidant defense gene *(MnSOD)* showed a difference in expression pattern in the ionomycin treatment groups compared with those in the control group. To begin with, although there was no statistical difference in the expression levels of Cx43 among experimental groups, the slight increase in Cx43 expression in the repeated ionomycin treatment groups is an indication of the pivotal role of Cx43 in early embryo development. In a previous study, it was reported that depletion of Cx43 expression in embryos brought about a decline in the rate of embryos reaching the blastocyst stage, which is consistent with our results [32].

Bax expression levels were slightly lower in Groups 2 and 3 compared with those in the control group, indicating that *Bax* expression is a useful indicator of blastocyst quality. *Bcl-2* and *MnSOD* increased markedly in Group 3, which suggests that repetitive ionomycin treatment leads to improved embryo viability. *MnSOD*, an antioxidant enzyme, functions to protect the mitochondrial components from superoxidation in the intracellular system [33]. Generally, it is considered that stimulated levels of the enzyme have evolved to address increased free-radical production during inflammatory episodes. The high *MnSOD* expression levels found in our results were consistent with those in previous reports that suggested that increased levels of *MnSOD* might be related to inadequate reprogramming of the donor genome in NT embryos [34–36]. However, the high expression levels of *MnSOD* in Group 3 might have been due to these embryos' increased vitality. Pursuant to one of the supportive reports, the increased *MnSOD* expression levels in *in vivo* bovine embryos and surviving *in vitro* embryos after vitrification bolster the theory that developmentally viable embryos have higher *MnSOD* expression [37]. In this respect, we suggest that cloned embryos exposed to an artificial environment during the *in vitro* process might have had to produce *MnSOD* to protect their viability. This was also supported by the fact that there was no significant difference in *MnSOD* expression levels between IVF treatment groups and the NT control sample, but there were improved developmental rates in the experimental groups, and increased expression levels of *Bcl-2* in Groups 2 and 3.

In conclusion, repetitive treatment with transient ionomycin can be effective in improving *in vitro* blastocyst development in bovine SCNT, as demonstrated by the varying expression patterns of apoptosis-related genes (*Bax, Bcl-2*, and *MnSOD*) in experimental treatment groups. Further studies will be needed to show whether or not multiple ionomycin activation with a shorter duration will improve the efficiency of cloned animal production.

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