FULL PAPER *Theriogenology*

Parthenogenetic Development and Ploidy following Various Chemical Activation Regiments of Bovine Oocytes

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ABSTRACT. Bovine oocytes treated using various combinations of 5 μM ionomycin (Ion), 10 μg/m*l* cycloheximide (CHX) and 5 μg/m*l* cytochalsin B (CCB) were evaluated to determine developmental rates and ploidy status. The groups were comprised of metaphase II oocytes exposed to Ion for 5 min (Group 1); Ion treatment followed by CHX for 5 hr (Group 2); Ion treatment followed by CHX/CCB for 1 hr and CHX for 4 hr (Group 3); Ion treatment followed by CHX/CCB for 3 hr and CHX for 2 hr (Group 4); and Ion treatment followed by CHX/CCB for 5 hr (Group 5). Group 5 exhibited significantly ($P<0.05$) lower rates of second polar body (PB) extrusion and higher rates of cleavage and blastocyst development. At 8 hr after Ion treatment, the eggs in groups 2, 3 and 5 were divided into 2 subgroups based on the presence or absence of the second PB and were assessed for cleavage rate and ploidy at the two-cell stage. The cleavage rates did not differ among the activation treatments or between the presence and absence of the second PB in all groups. The diploid rate was significantly ($P<0.05$) higher in group 5 than in groups 2 and 3. However, the diploid rate in blastocyst-stage parthenotes did not differ among groups 2, 3 and 5. Consequently, oocyte activation by CHX/CCB for 5 hr after Ion treatment could enhance diploid parthenogenetic development in bovine.

KEY WORDS: bovine parthenote, chemical activation, ploidy, polar body.

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In bovine, oocyte activation is a major factor for successful production of reconstructed embryos by somatic cell nuclear transfer (SCNT) [34], parthenogenesis and intracytoplasmic sperm injection [23]. During normal fertilization in mammalian species, sperm penetration into the oocyte triggers intracellular Ca^{2+} oscillation followed by inactivation of the maturation promoting factor (MPF) composed of the catalytic subunit of cdc2p34 protein kinase and the regulatory subunit cyclin B1/B2 for about 2 hr [37]. Although artificial stimuli, such as Ca^{2+} ionophore [19], ionomycin [23], ethanol [31], electric pulse [27] and strontium ($SrCl_2$) [21] can activate bovine oocytes by increasing intracellular Ca^{2+}, the efficiency of artificial stimuli is limited [25, 34]. Therefore, additional chemical agents, such as cycloheximide (CHX, protein phosphorylation inhibitor) [24] and 6-dimethylaminopurine (DMAP, protein synthesis inhibitor) [29, 30], that prevent MPF re-accumulation by reducing cdc2 kinase activity [2] have also been applied.

DMAP treatment of metaphase II oocytes induces diploid activation by preventing chromosomal separation and extrusion of the second polar body (PB) [19]. However, CHX does not prevent chromosomal segregation and second PB extrusion, resulting in haploid development. When cytochalasin D (CCD) was added in the medium, chromosomal segregation manifested, but the cytokinesis that induces second PB extrusion was prohibited in CHX-treated oocytes, leading to diploid development with two pronuclei in activated oocytes [4, 19]. The combined treatment of ionomycin with DMAP for oocyte activation could induce an abnormal pattern of karyokinesis during the first cell cycle [8], and thus, CHX has widely been used as an alternative approach [19]. Moreover, in cloning, oocyte activation using CHX produces healthy live offspring in cattle [12, 17], but the live offspring rate is still low [5].

Campbell *et al.* [3] proposed the hypothesis that when donor cells at G1 stage are reconstructed with MII oocytes that maintain a high level of MPF, the resulting embryos have chromosomal abnormalities or changes in ploidy during the first cell cycle. Although eggs reconstructed with G1 stage nuclei that possesses 2 chromatins could replicate their DNA and extrude the PB, and formation of 2 chromatins resulted in a failure of normal ploidy. This phenomenon has been observed in mice [6, 32, 33] and to some extent is exhibited in bovine and pig [33]. However, attempts have been made to produce cloned bovine embryos using CHX treatment combined with cytochalasin B (CCB) or D (CCD) for 1 hr, 5 hr or 6 hr in order to prevent extrusion of pseudo PBs from reconstructed eggs with G0/G1 stage nuclei [12, 17]. A similar study has also been carried out in porcine using CCB treatment after electric or chemical oocyte activation [22]. The major functions of CCB include blockage of cytoplasmic cleavage followed by prevention of formation of contractile microfilament structures, which inhibits uptake and incorporation of precursor into the mucopolysaccharides and glycoproteins of the cell affecting its membrane biosynthesis, resulting in multinucleate cell formation, reversible inhibition of cell movement and induction of nuclear extrusion [16, 19].

Generally, *in vitro* produced embryos show high incidence of chromosomal abnormalities at the early stages. However, as the development stage progresses, the incidence decreases gradually [13, 15]. This implies the need to

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analyze the specific effect of chemical activation agents on the ploidy of embryos both at early and later stages instead of examining only at the blastocyst stage. Based on the above facts, the present study was designed to evaluate the effects of CCB with 5 hr CHX treatment after ionomycine treatment, based on commonly used methods [12, 17, 24], on the parthenogenetic development and ploidy of early and later stages of bovine embryos.

MATERIALS AND METHODS

Chemicals and media: All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and all media were purchased from Gibco (Invitrogen Corporation, Breda, Netherlands), unless otherwise specified. The medium used for oocyte maturation was M199 containing Earle's salts, 10% fetal bovine serum (FBS), 25 mM hepes, 2.5 mM Na- pyruvate, 1 mM L-glutamine, 50 μg/m*l* gentamycin (BioWhittaker, Walkersville, MD, U.S.A.), 10 μg/m*l* FSH and 10 μg/m*l* LH. Tyrode's lactate hepes (114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.4 mM NaH_2PO4H_2O, 2 mM $CaCl_2 2H_2O$, 0.5 mM $MgCl_2 6H_2O$, TL-hepes) medium supplemented with 3 mg/m*l* bovine serum albumin (BSA, essential fatty acid free, Fraction V), 10 mM Na lactate and, 10 mM hepes was used for preparation of 5 μg/m*l* Ion and washing of oocytes. The medium used for *in vitro* culture (IVC) of parthenotes was synthetic oviduct fluid medium (SOF) supplemented with 10 μl/m*l* modified eagle's medium (MEM) non-essential amino acids, 0.4 mM Na pyruvate, 1 mM L-glutamine, 8.4 mg/m*l* BSA, 50 μg/m*l* gentamycin and 2.92 μg/m*l* ethylene diamine tetra acetic acid (EDTA; SOF-A) or 20 μl/m*l* modified eagle's medium (MEM) amino acids (SOF-B). SOF-A was used for preparation of 5 μg/m*l* CCB and 10 μg/m*l* CHX. The pH of all media was adjusted to 7.4, and osmolality was adjusted to 280 mOsm/kg.

Oocyte preparation: Bovine ovaries collected from a local abattoir were transported within 2 hr to the laboratory in phosphate buffered saline (PBS) containing 100,000 IU/*l* penicillin and 100 mg/*l* streptomycin at approximately 30°C. Cumulus-oocyte complexs (COCs) were aspirated from follicles 2 to 7 mm in diameter using an 18-G needle attached to a 10-m*l* syringe and were subsequently rinsed twice in TL-hepes medium. Morphologically healthy COCs with an intact cumulus investment and homogenous cytoplasm were selected for *in vitro* maturation (IVM).

Sets of 15 COCs were matured in 50 μl droplets of IVM medium at 39°C in a humidified atmosphere of 5% CO_2 in air for 22 hr. After IVM, the expanded COCs were vortexed for 1 min in TL-hepes medium supplemented with 1% hyaluronidase in order to remove their cumulus cells and rinsed twice in fresh TL-hepes.

Oocyte activation protocol: Denuded oocytes were screened under an inverted microscope (× 400), and those with the first PB and dense cytoplasm were selected and randomly assigned to 5 chemical activation treatment groups. Group 1: Oocytes were exposed to 5 μM ionomycin (Ion) for 5 min (Group 1). Group 2: Oocytes were exposed to Ion for 5 min and cultured in 10 μg/m*l* CHX for 5 hr. Group 3: Oocytes were exposed to Ion for 5 min, initially cultured in 10 μg/m*l* CHX combined with 5 μg/m*l* CCB for 1 hr and then cultured in 10 μg/m*l* CHX for 4 hr. Group 4: Oocytes were exposed to Ion for 5 min, initially cultured in 10 μg/m*l* CHX combined with 5 μg/m*l* CCB for 3 hr and then cultured in 10 μg/m*l* CHX for 2 hr. Group 5: Oocytes were exposed to Ion for 5 min and cultured in 10 μg/m*l* CHX combined with 5 μg/m*l* CCB for 5 hr. Immediately after Ion treatment, the eggs of all groups were rinsed in TL-hepes medium containing 30 mg/m*l* BSA for 5 min to stop activation.

In vitro culture (IVC): All activated eggs were rinsed twice in fresh SOF-A medium, cultured in sets of 30 in 30 μl drops of SOF-A medium until 72 hr and subsequently cultured in SOF-B at 39°C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for up to 216 hr. At 120 hr, the cultures were "fed" 10 μl of fresh SOF-B medium. Parthenotic development was assessed under an inverted microscope at 24 hr intervals for up to 216 hr post activation (hpa). Cleavage and blastocyst rates were assessed at 48 and 216 hpa, respectively.

Cytological analysis: The ploidy status of parthenotes was evaluated as described previously by King *et al.* [14]. In brief, the cell stages of two-cell and blastocyst stage parthenotes were synchronized at metaphase by addition of 0.05 μg/m*l* demceolcine for 8 hr and 3 hr, respectively. The parthenotes were then treated with 0.5% trypsin for 5 min, transferred into 0.8% Na citrate solution for 5 min and fixed overnight in methanol:acetic acid (3:1). The fixed parthenotes were mounted onto a pre-cleaned microscope slide, and methanol:acetic acid (1:1) solution was dropped onto them for preparation of spreads. The slides were air dried and stained with 4% Giemsa solution (w/v) for 5 min. Ploidy was evaluated under a light microscope with oil-immersion optics (1,000 ×), and parthenotes were classified as aneuploid, haploid, diploid, tetraploid, polyploid and mixoploid.

Experimental design: Experiment 1 assessed the efficacy of chemical activation treatments in relation to the second PB extrusion rate. In 4 replicates, the second PB extrusion rates of 430 oocytes activated using five different activation treatments were assessed at 8 hpa. Experiment 2 assessed the efficacy of chemical activation treatments in relation to parthenogenetic development. A total of 536 oocyte in 5 replicates were exposed to 5 different activation treatments, and their cleavage, blastocyst and hatched blastocyst rates were assessed at 48, 192 and 216 hpa, respectively. The results of this experiment formed the basis for the subsequent experiments. Experiment 3 assessed the efficacy of presence or absence of a second PB on the cleavage rate and ploidy status of parthenotes at the 2-cell stage. In 4 replicates, a total of 239 oocytes from activation groups 2, 3, and 5 were further divided into two subgroups based on the presence or absence of a second PB, respectively, in order to evaluate the cleavage rate at 48 hpa. The ploidy statuses of

parthenotes at the two-cell stage from all the groups were assessed. Experiment 4 assessed the efficacy of chemical activation treatments in relation to ploidy status in parthenotes at the blastocyst stage. The ploidy statuses of a total 50 parthenotes at the blastocyst stage from activation groups 2, 3 and 5 were analyzed at 216 hpa.

Statistical analysis: Differences in development rates among activation groups were analyzed using one-way ANOVA by SPSS 10.0 (SPSS Inc., Chicago, IL, U.S.A.). Comparisons of means among groups were performed using the Duncan's multiple range test and Tukey multiple comparisons test. Values were expressed as means ± standard deviation (SD). Differences were considered significant when $P<0.05$.

RESULTS

Effect of chemical activation treatments on the second PB extrusion rate: In a preliminary experiment, nuclear activation of oocytes in groups 1 and 2 was estimated (Data was not shown) 19 hr after Ion treatment. The results were 69.2% (83/120) and 94.8% (128/135) in groups 1 and 2, respectively. The second PB extrusion rates of oocytes treated using 5 different parthenogenetic activation protocols were assessed at 8 hpa (Fig. 1). Among the groups, the second PB extrusion rate of the parthenote eggs the in group 5 (22.0 ± 4.6) was significantly ($P<0.05$) lower than those of the other groups. The parthenote eggs in group 2 exhibited a relatively high (67.1 ± 8.4) extrusion rate for the second PB compared with groups 1, 3, and 4, but the rate did not differ significantly among the groups.

Effect of chemical activation treatments on parthenogenetic development: The effects of chemical activation of bovine oocyte on parthenogenetic development in terms of cleavage, blastocyst and hatched blastocyst rates at 48, 192 and 216 hpa, respectively, are presented in Table 1. The cleavage rate (19.7 ± 10.4) of group 1 was significantly ($P<0.05$) lower than those of the other groups, but the rate did not differ significantly among groups 2–5. The blastocyst development rate of group 1 (0.8 ± 1.8) was significantly ($P<0.05$) lower than those of the other groups. Among groups 2–5, a significantly ($P<0.05$) higher blastocyst development rate (30.9 ± 9.3) was observed in group 5. None of the blastocysts in groups 1–4 developed to hatched blastocyst, whereas 5 (16.1%) out of 31 blastocysts in group 5 developed to hatched blastocysts. Since no significant difference was observed in relation to second PB extrusion and development to cleavage and blastocyst between groups 3 and 4, group 4 was excluded from subsequent experiments. Likewise, group 1, which exhibited very low developmental competence, was also excluded.

Effect of presence or absence of the second PB on the cleavage rate and ploidy status of parthenotes at the 2-cell stage: The cleavage rates of oocytes in groups 2, 3 and 5 were evaluated based on the presence or absence of the second PB at 48 hpa (Fig. 2). No significant differences were observed in the cleavage rates either among the different activation treatments or between with or without a PB in any respective group.

The chromosome statuses of the two-cell stage parthenotes in groups 2, 3 and 5 based on the presence or absence of the second PB are presented in Table 2. In group 2, 2-cell stage parthenotes derived from oocytes with a second PB, the frequencies of diploid (Fig. 3b), haploid and aneuploid parthenotes were 43.8%, 43.8% and 12.5%, respectively. On the other hand, a low haploid frequency (25.0%, 2/8; Fig. 3a), relatively high diploid frequency (62.5%, 5/8) and 1 tetraploid (Fig. 3c) were observed in par-

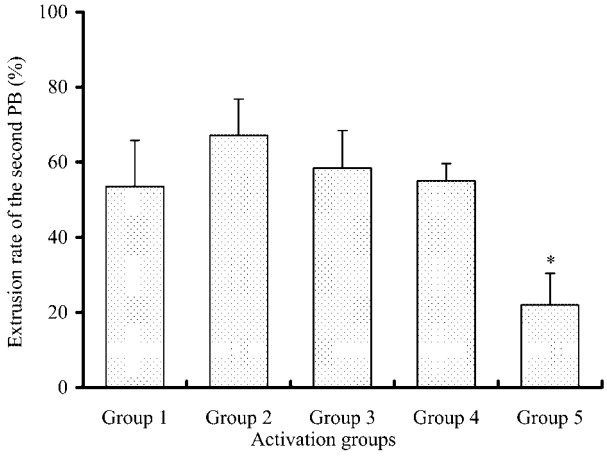

Fig. 1. Extrusion rates of the second polar bodies of oocytes following different activation treatments. Group 1, 5 μM Ion for 5 min; Group 2, 5 μM Ion for 5 min+10 μg/m*l* CHX for 5 hr; Groups 3 and 4, 5 μM Ion for 5 min+10 μg/m*l* CHX and 5 μg/m*l* CCB for 1 hr or 3 hr+10 μg/m*l* CHX for 4 or 2 hr, respectively; Group 5, 5 μM Ion for 5 min+10 μg/m*l* CHX and 5 μg/m*l* CCB for 5 hr. Error bars indicate the standard deviation.

Table 1. Development of parthenotes following different activation treatments

Groups*	Oocytes used	Development rate (%, mean ± SD)		Hatched blastocyst (%)
		Cleavage	Blastocyst	
1	105	20 (19.7 ± 10.4)[a)]	1 (0.8 ± 1.8)[a)]	0 (0)
2	113	66 (58.7 ± 18.1)[b)]	7 (6.2 ± 2.4)[b)]	0 (0)
3	121	69 (54.0 ± 23.0)[b)]	17 (13.8 ± 4.1)[c)]	0 (0)
4	97	51 (53.5 ± 15.8)[b)]	12 (12.6 ± 6.4)[bc)]	0 (0)
5	100	68 (67.7 ± 12.8)[b)]	31 (30.9 ± 9.3)[d)]	5 (3.8)

Five Replicates.

* Group 1, 5 μM Ion for 5 min; Group 2, 5 μM Ion for 5 min + 10 μg/m*l* CHX for 5 hr; Groups 3 and 4, 5 μM Ion for 5 min + 10 μg/m*l* CHX and 5 μg/m*l* CCB for 1 hr or 3 hr + 10 μg/m*l* CHX for 4 or 2 hr, respectively; Group 5, 5 μM Ion for 5 min + 10 μg/m*l* CHX and 5 μg/m*l* CCB for 5 hr.

a-d) Percentages with different superscripts within columns are significantly different ($P<0.05$) .

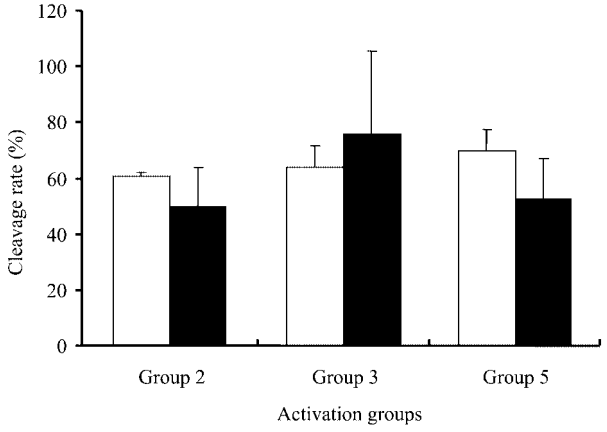

Fig. 2. Cleavage rates of parthenotes with or without a second polar body 48 hr after oocyte activation. Group 2, 5 μM Ion for 5 min + 10 μg/m*l* CHX for 5 hr; Group 3, 5 μM Ion for 5 min + 10 μg/m*l* CHX and 5 μg/m*l* CCB for 1 hr + 10 μg/m*l* CHX for 4; Group 5, 5 μM Ion for 5 min + 10 μg/m*l* CHX and 5 μg/m*l* CCB for 5 hr. Error bars indicate the standard deviation. Open bars represent eggs without a second polar body; solid bars represent eggs a second polar body.

thenotes without a second PB. In group 3, parthenotes derived from oocytes with a second PB exhibited a low haploid frequency (29.4%, 5/17) and high diploid frequency (64.7%, 11/7). On the other hand, in parthenotes derived from oocytes without a second PB, the majority of parthenots were diploid (77.8%, 7/9), and none were haploid. In group 5, the ploidy status of parthenotes from oocytes with a second PB could not be analyzed as only some of them developed to the two-cell stage. However, 10 parthenotes were analyzed for their ploidy status, but their ploidy statuses could not be ascertained. In contrast, the majority (83.3%) of parthenotes derived from oocytes without a second PB were diploid. None were categorized as tetraploid in group 3, and none were categorized as haploid or aneuploid in group 5.

Statistical by comparison of the overall status of ploidy among the activation groups revealed a significantly ($P<0.05$) higher haploid frequency in group 2 (37.8 ± 3.9) than in group 3 (19.2 ± 12.4). On the other hand, the diploid frequency was significantly ($P<0.05$) higher in group 5 (87.8 ± 10.7) than in groups 2 (45.2 ± 9.0) and 3 (68.6 ± 22.9), but there was no difference between groups 2 and 3.

Effect of chemical activation treatments on ploidy status in parthenotes at the blastocyst stage: The ploidy statuses as analyzed by examination of 250 chromosomal spreads from 50 day-9 parthenotes developed to the blastocyst stage in groups 2 (n=7), 3 (n=15) and 5 (n=28) are presented in Table 3. The diploid frequency was higher in group 2 (100%) than in groups 5 (70.8%) and 3 (50%). Haploidy was only observed in group 3 (8.3%). Mixoploidy and polyploidy were observed in groups 3 (33.3% and 8.3%, respectively) and 5 (20.8% and 8.3%, respectively).

Table 2. Ploidy analysis of parthenotes at the 2-cell stage

Groups*	Total number of embryos analyzed		Number (%)			
			Haploid	Diploid	Tetraploid	Aneuploid
2	1 PB	8	2 (25.0)	5 (62.5)	1 (12.5)	0 (0)
	2 PB	16	7 (43.8)	7 (43.8)	0 (0)	2 (12.5)
	Total (mean ± SD)	24	9 (37.8 ± 3.9)[c)]	11 (45.2 ± 9.0)[a)]	1 (6.7 ± 11.5)	3 (10.4 ± 10.0)
3	1 PB	9	0 (0)	7 (77.8)	0 (0)	2 (22.2)
	2 PB	17	5 (29.4)	11 (64.7)	0 (0)	1 (5.9)
	Total (mean ± SD)	26	5 (19.2 ± 12.4)[b)]	18 (68.6 ± 22.9)[a)]	0 (0 ± 0)	3 (12.2 ± 11.3)
5	1 PB[†]	18	0 (0)	15 (83.3)	3 (16.7)	0 (0)
	Total (mean ± SD)	18	0 (0 ± 0)[a)]	15 (87.8 ± 10.7)[b)]	3 (12.2 ± 10.7)	0 (0 ± 0)

Four replicates.
* Group 2, 5 μM Ion for 5 min + 10 μg/m*l* CHX for 5 hr; Group 3, 5 μM Ion for 5 min + 10 μg/m*l* CHX and 5 μg/m*l* CCB for 1 hr + 10 μg/m*l* CHX for 4; Group 5, 5 μM Ion for 5 min + 10 μg/m*l* CHX and 5 μg/m*l* CCB for 5 hr.
One PB means embryos without the second polar body.
Two PB means embryos with the second polar body.
a) Two PB was not analyzed because only some developed.
b,c) Percentages with different superscripts within columns are significantly different ($P<0.05$).
The numbers of embryos and metaphase spreads analyzed in each group were too small to compare the percentages of ploidy, respectively.

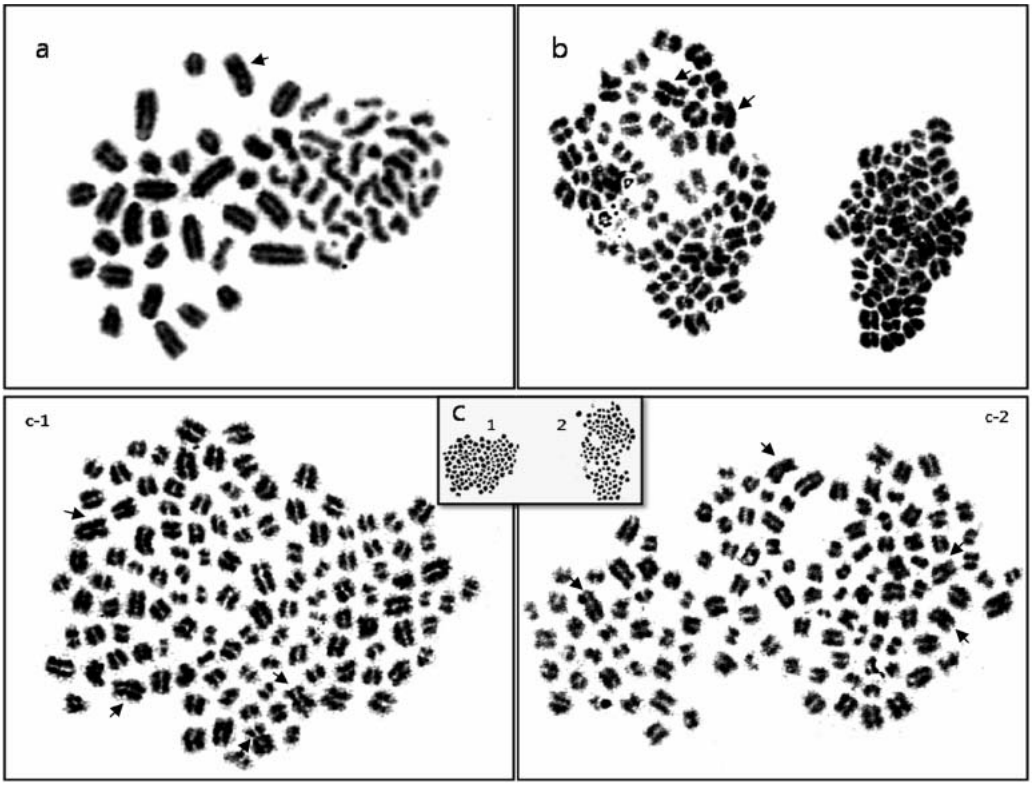

Fig. 3. Chromosome spreads of two-cell parthenotes treated with ionomycin followed by CHX for 5 hr. (a) Haploid chromosome set of a parthenote without a second PB (1,000 ×). The X-chromosome from the haploid chromosome set of the right blastomere could not be distinguished because the chromosome set did not condense enough. (b) Diploid chromosome sets of a parthenote with a second PB (1,000 ×). The X-chromosome from the diploid chromosome set of the right blastomere could not be distinguished because there was excess condensation of the chromosome set. (c) Tetraploid chromosome sets of a parthenote without a second PB (400 ×). (c-1) and (c-2) Display of each chromosome set in c at high magnification (1,000 ×). Arrows indicate the X- chromosome.

Table 3. Ploidy analysis of day-9 parthenotes at the blastocyst stage

Groups*	Number of blastocysts analyzed	Number of blastomeres spread	Ploidy (%)				Unknown (%)
			Haploid	Diploid	Mixoploid	Polyploid	
2	7	23		4 (100)			3 (42.8)
3	15	77	1 (8.3)	6 (50)	4 (33.3)	1 (8.3)	3 (20)
5	28	150		17 (70.8)	5 (20.8)	2 (8.3)	4 (14.3)

* Group 2, 5 μM Ion for 5 min + 10 μg/m*l* CHX for 5 hr; Group 3, 5 μM Ion for 5 min + 10 μg/m*l* CHX and 5 μg/m*l* CCB for 1 hr + 10 μg/m*l* CHX for 4; Group 5, 5 μM Ion for 5 min + 10 μg/m*l* CHX and 5 μg/m*l* CCB for 5 hr.
The numbers of embryos and metaphase spreads analyzed in each group were too small to compare the percentages of ploidy, respectively.

DISCUSSION

During parthenogenetic development, mammalian oocytes undergo the second meiotic division by calcium releasing stimulus with various agents, such as exogenous calcium, electric shock and enzymes. They resume the mitotic cell cycle due to subsequent exposure to protein synthesis inhibitors [7] or broad-spectrum protein kinase inhibitors [30]. Enhancement of the developmental competence of bovine oocytes has been reported by the use of cytochalasin B (CCB), which may prevent extrusion of the second PB, resulting in diploid development [9]. The present study was designed to evaluate the effect of Ion, CHX and the combination of CHX with CCB on the developmental rates and ploidy of bovine parthenote embryos. The results indicated that an extended treatment of CCB in combination with CHX for 5 hr effectively decreased extrusion of the second PB, increased the diploid status at the two-cell and blastocyst stages and enhanced the developmental rate of bovine parthenotes. In addition, activation with CHX alone after Ion treatment resulted in relatively high diploid and low haploid statuses at the two-cell stage.

One of the key factors responsible for successful SCNT is the cell cycle synchronous transplantation between donor nuclei and cytoplasm, and thus, extensive use of G0/G1 somatic cells have resulted in improvement of the development of cloned embryos and birth of offspring [11, 35, 38]. CCB and CCD have been widely used to prevent extrusion of pseudo PBs of G0/G1 somatic donors during the SCNT procedure [3]. With a similar purpose, exposure to these chemicals at different times, such as 1, 5 or 6 hr, have also been attempted in bovine [1, 11, 20, 24, 38]. In the present study, groups 1–4 showed extrusion rates ranging from 53–67%, whereas group 5 exhibited a significantly low rate of 22%. Treatment with a combination of CHX/CCB for 1 or 3 hr did not produce any difference in the rates of extrusion of the second PB. In mammals, extrusion of the second PB occurs at about 5 hr after fertilization with sperm or following stimulation with activators [32, 33]. However, CCB treatment for 1–3 hr could not completely block extrusion of the second PB. Our result is consistent with the previous report of Liu *et al.* [19], which showed 85% and 12% extrusion of the second PB from parthenotes treated using CHX with or without CCD for 6 hr after Ca^{2+} ionophore treatment, respectively. Based on the above observations, we concluded that activation of oocytes with CCB for a minimum of 5 hr is required to prevent extrusion of the second PB.

In parthenote embryos, confirmation of embryonic developmental competence before implantation is very important because growth arrest of most haploid parthenote embryos occurs at early stages and only a few embryos reach the blastocyst stage. On the other hand, dipioid parthenote embryos have been known to possess similar developmental competence to IVF embryos [18, 28]. Higher developmental rates were associated with groups activated by combination of CHX/CCB compared with treatment of Ion and CHX alone, but there were no differences between groups 1 and 3. In addition, CHX/CCB treatment for 5 hr effectively improved the development of bovine parthenotes, and moreover, only 5 of 100 oocytes developed to hatached blastocysts in this group (Table 1). Liu *et al.* [19] reported 27% blastocyst rates using CHX combined with 2.5 μg/m*l* CCD for 6 hr after Ca^{2+} ionophore treatment, but they could not obtain development to the hatched blastocyst stage. Presicce and Yang [24] also reported that the combination of CHX with CCB after ethanol treatment could improve the development of bovine parthenotes. Improved development after treatment with CCB or CCD may be due to their ability to induce diploid parthenotes by preventing cytokinesis, and formation of diploid embryos is similar to that of the normal fertilization process, thus, resulting in better developmental rates [19, 24].

The cleavage rate is considered the standard for assessing the early developmental capacity of embryos. Hence, the cleavage rates were compared at 48 hr to examine the effect of extrusion of the second PB in different activation groups. The results showed that the rate of cleavage was the unaffected by the status of extrusion of the second PB and the different activation treatments in the parthenote embryos.

Despite many reports on ploidy analysis, only a very few have been done in the early stages of embryos in the bovine. Slimane *et al.* [26] reported that 34.2% of two-cell IVF embryos had abnormalities such as haploidy, mixoploidy, hyperploidy and hypoploidy. Previous studies have examined pronuclear formation status and PB extrusion to judge the presumptive nucleus state [19, 25, 29]. However, pronuclear formation may not truly indicate the ploidy of activated oocytes [2]. Moreover, evaluating the effect of a chemical agent through ploidy analysis of blastocysts may

often be misleading, because most embryos with abnormal ploidy gradually undergo growth arrest or death during embryonic development and thus cannot be assessed. Research in past years has shown that embryos might selectively eliminate cells with abnormal karyotypes depending on the developmental stage or might possess mechanisms to remove chromosomally abnormal cells by programmed cell death or apoptosis [8, 10, 15]. In view of this, our study analyzed ploidy in two-cell stage parthenote embryos to obtain insight into the influence of chemical agents immediately after completion of the first cell cycle. The results indicate that prolonged activation with CHX in combination with CCB increased the number of diploid parthenotes while reducing the number of haploid parthenotes. In addition, activation with CHX alone after Ion treatment appeared to induce a high diploid rate, as reported previously by Winger *et al.* [36]. From the above results, it can be suggested that although CCB prevented extrusion of the second PB, treatment for 1 or 3 hr could not completely block extrusion. On the other hand, CHX could often prevent chromosomal segregation or extrusion of the second PB. This was evidently shown by formation of over 80% diploid eggs following the combined treatment with CHX and CCB for 5 hr. Two-cell parthenotes pretreated with CHX alone for 5 hr not only showed diploidy with a second PB but also haploidy and tetraploidy without a second PB. This may be because a nonspecific chemical agent like CHX affects several metabolic pathways and induces chromosomal abnormalities by preventing PB extrusion and premature DNA synthesis [2].

Although a few reports are available on ploidy analysis of both early and late stage parthenogenic embryos in bovine [36], there is a lack of information on ploidy status following activation by CHX with or without CCB. The present study did not directly compare early and late stage parthenogenic embryos, but the blastocysts obtained by CHX with or without CCB treatment on day 9 showed extremely lower rates of haploidy and increased rates of mixoploidy compared with the two-cell parthenotes. Moreover, only a few parthenote embryos treated with CHX alone after Ion treatment developed to blastocysts, and they were all diploid. We presume that haploid parthenotes often undergo developmental arrest at an early stage. On the other hand, diploid parthenotes might possess developmental competency similar to IVF embryos [18, 36], and hence, they could develop to the blastocyst stage. All blastocysts developed from eggs treated with CCB exhibited mixoploidy, and this phenomenon has often been observed in IVF embryos. This might arise through fusion of blastomeres in growing embryos or through chromosome replications without subsequent karyokinesis during cleavage [13].

In conclusion, oocyte activation using CHX in combination with CCB for a minimum duration of 5 hr after Ion treatment could enhance parthenogenetic development and produce higher rates of diploidy by preventing extrusion of the second PB in bovine. Activation by CHX alone to produce haploid parthenotes was not ideal because the majority of 2-cell and blastocyst parthenotes were diploid and a few were tetraploidy. Furthermore, based on our results, it can be suggested that activation by CHX combined with CCB for 5 hr can be employed for reconstruction of embryos using a G0/G1 donor cell and MII oocyte in the process of cloning. However, further studies are needed to identify a more effective and specific chemical for MPF stabilization.

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