Journal of Reproduction and Development, Vol. 49, No. 2, 2003

—Original—

Parthenogenetic Activation and Subsequent Development of Porcine Oocytes Activated by a Combined Electric Pulse and Butyrolactone I Treatment

YinZhong BING[1,3], Limei CHE[1,2], Yuji HIRAO[1], Naoki TAKENOUCHI[1], Heriberto RODRIGUEZ-MARTINEZ[4] and Takashi NAGAI[5]

[1]National Agriculture Research Center for Tohoku Area, Morioka, Iwate 020–0198, Japan, [2]Tokyo University of Agriculture, Tokyo 156–8502, Japan, [3]Northeast Institute of Geography and Agricultural Ecology, Chinese Academy of Sciences, Harbin 150040, China, [4]Swedish University of Agricultural Sciences (SLU), P. O. Box 7039, SE-750 07 Uppsala, Sweden, [5]National Institute of Agriobiological Sciences, Tsukuba, Ibaraki 305–8602, Japan

Abstract. This study was designed to evaluate the parthenogenetic activation of porcine oocytes matured *in vitro* for a varied period after combined electric pulse (EP; 1500 V/cm, 100 μsec) and Butyrolactone I (BL I). After 36 h of maturation culture, the rates of activated oocytes and oocytes with two pronuclei were significantly lower than those of oocytes cultured for 42 and 48 h after EP. However, when treated by a combined EP and BL I (150 μM), these rates increased to the same level as 42 and 48 h oocytes. When oocytes cultured for 48 h and activated by a combined EP and BL I treatment were subsequently cultured in mNCSU37 medium, the rates of embryos cleaved and developed to the blastocyst stage were significantly higher than those in Whitten's medium. In contrast, when activated oocytes were cultured in mNCSU37 medium under two oxygen environments (5% vs 20% O_2), there was no difference in the rates of cleavage, blastocyst formation and nuclear numbers per blastocyst. Our results demonstrated that the combined EP and BL I treatment of porcine oocytes matured *in vitro* is capable of producing high rates of good quality blastocysts when cultured in a suitable *in vitro* condition.

Key words: Porcine oocyte, Blastocyst, Activation, Electric pulse, Butyrolactone I

(**J. Reprod. Dev. 49:** 159–166, 2003)

Mammalian follicular oocytes can be matured *in vitro*. In oocytes matured *in vivo* and *in vitro*, development is arrested at the metaphase II (M-II) stage until insemination or parthenogenetic activation. This process is mainly regulated by the cytoplasmic maturation promoting factor (MPF), a complex of cyclin and $p34^{cdc2}$, which is stabilized by cytostatic factor (CSF) [1, 2]. Under natural conditions, oocyte activation is induced by the sperm penetrating the oocytes and inducing the oscillation of intracellular levels of free calcium ions (Ca^{2+}) [3]. The elevated cytoplasmic Ca^{2+} levels influence the activity of the MPF [4] and the CSF [5], and result in the developmental progress of oocytes from the M-II stage to interphase [6]. Artificial stimuli, such as exposure to ethanol, the Ca^{2+} ionophore A 23187, or direct electric pulses (EP), can elevate the cytoplasmic Ca^{2+} levels and cause oocyte activation. However, in parthenogenetic activation of porcine oocytes, these activation treatments seem to be unable to provide an adequate or full-valued activation [7, 8] or to

Accepted for publication: January 20, 2003
Correspondence: T. Nagai

suppress synthesis of the proteins responsible for maintenance of the oocytes at the M-II stage [6, 9, 10]. Therefore, the search for more suitable methods of activation for porcine oocytes, for example, a combination of Ca^{2+} elevating reagents with either various inhibitors of protein kinases or inhibitors of protein synthesis, would be desirable.

The development capacity of activated porcine oocytes was not only affected by different activation methods [9, 11–13], but also strongly affected by the quality of oocytes [14–17]. Usually, porcine follicular oocytes reach the M-II stage after 36 h of *in vitro* maturation (IVM) culture [14–19], but newly matured porcine oocytes (36 h of maturation culture) showed lower developmental potentials after *in vitro* fertilization (IVF) compared to the oocytes cultured for 44 h [20] or after parthenogenetic activation of oocytes cultured for 42 or 48 h [16, 17]. However, a prolonged period of maturation culture of porcine oocytes at the M-II stage may cause deterioration of various cytoskeletal components [15, 21]. Selection of a suitable maturation culture period of porcine oocytes is expected to enhance oocyte activation and subsequent embryo development.

Butyrolactone I (BL I), a specific inhibitor of the cyclin-dependent kinases (cdks), acts as a competitive inhibitor of ATP [22]. BL I can induce a sharp decrease in MPF activity and subsequent reduction in histone H1 kinase activity when it was added to the M-II stage of porcine oocytes [23]. We have previously reported that BL I can inhibit meiotic resumption in oocytes that are in the germinal vesicle (GV) stage by reducing H1 kinase activity [24] or induce activation in oocytes that are in the M-II stage [25] in pigs. However, the activation and the subsequent development of porcine oocytes at various periods of maturation culture have not been tested. In addition, although the achievements made early stages of embryonic development feasible by parthenogenetic activation, the suitable culture conditions for porcine embryos have yet to be determined.

The objective of the present study was to assess efficiency of oocyte activation by a combined treatment of EP and BL I on porcine oocytes after various periods of maturation culture, and to define culture conditions that provide the best *in vitro* environment for development of early porcine embryos obtained from activated oocytes under a defined maturation culture period: a) two different culture media, modified Whitten and NCSU37 media and b) two different gas compositions, 5% and 20% oxygen tension atmospheres were evaluated.

Materials and Methods

Oocytes collection and in vitro maturation (IVM)

Porcine follicular oocytes were collected as described in a pervious study [26]. Ovaries were collected from pre-pubertal gilts (mainly Landrace × Large White × Duroc) at a local slaughterhouse and transferred to the laboratory in a warm insulated flask (25–27 C). The ovaries were then washed with 0.2% cetylmethylammonium bromide (Wako, Osaka, Japan) and rinsed 3 times with Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline [PBS (–)] that contained 0.1% polyvinyl alcohol (PVA, Sigma, St. Louis, MO, USA). The cumulus-oocyte complexes (COCs) were aspirated from the antral follicles, 3 to 6 mm in diameter through an 18-gauge needle attached to a 10 ml syringe. Only those oocytes surrounded by several layers of unexpanded cumulus cells and homogeneous ooplasm were selected and these were washed twice with modified Tyrode s solution (TL-Hepes) and twice with the final maturation medium. The maturation medium was modified TCM199 (with Earle's salt and 25 mM Hepes; Gibco, NY, USA) supplemented with 10% (v/v) heat-treated fetal calf serum (FCS; Biowest, USA), 0.1 mg/ml sodium pyruvate (Nakarai, Kyoto, Japan), 50 μg/ml gentamicin (Sigma), 1 μg/ml estradiol (Sigma), 10 IU/ml pregnant mare's serum gonadotropin (PMSG, Serotropin, Tokyo, Japan) and 10 IU/ml human chorionic gonadotropin (hCG, Teikokuzouki, Tokyo, Japan), and 150 μM cysteamine (Nakarai). Ten selected COCs were transferred to a droplet of 100 μl of maturation medium covered with paraffin oil (Nakarai) in a petri dish (FALCON, 1008, Bencton Dickison Labware, USA) and cultured at 38.5 C in a moist atmosphere of 5% CO_2 in air for 36, 42 and 48 h according to the requirement of different experiment designs.

Parthenogenetic activation of oocytes

After maturation culture, COCs with expended cumulus cells were treated with hyaluronidase (1 mg/ml; Sigma) and cumulus cells were removed

physically using a narrow pipette. Oocytes with a first polar body were selected and washed three times by mTCM199 supplemented with 4 mg/ml bovine serum albumin (BSA, Fractive V, Sigma), and then subjected to a single DC pulse (1,500 V/cm; ECM 200, BTX Inc., San Diego, USA) of 100 μsec in 0.3 M solution of mannitol (Wako, Osaka, Japan) containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.1% PVA (Sigma). After the electro-stimulated oocytes were washed 5 times by mTCM199 supplemented with 4 mg/ml BSA, they were incubated for 4 h in 20 μl drops of mTCM199 containing 4 mg/ml BSA and 5 μg/ml cytochalasin B (CB, Sigma), with or without 150 μM BL I as described in our previously study [25]. CB was added to the medium to produce activated oocytes with 2 pronuclei and 1 polar body (2PN1PB), because this diploid status is considered to be normal for parthenotes [27, 28].

Evaluation of nuclear status after maturation culture and activation of oocytes at various periods of IVM culture

Matured or activated oocytes at various periods of maturation culture were mounted on slide, fixed with acetic alcohol (1:3 v/v) for 48 h and stained with 1% aceto-orcein. The rates of oocytes that had underwent germinal vesicle break down (GVBD) and matured to the M-II were elevated. Activation was considered to have occurred if the oocytes were in the stages of premature chromosome condensation (PCC) and pronuclear (PN) formation. Activated oocytes with more than 3 pronuclei remaining were considered to be abnormal nuclear status. Oocytes with the first polar body and metaphase chromosomes were not considered to be activated.

Experimental design

In Experiment 1, to determine when oocytes were reaching M-II, oocytes were fixed and assessed for meiotic stages at 36, 42 and 48 h of IVM culture.

In Experiment 2, effects of the periods of maturation culture and the methods to induce parthenogenetic activation, oocytes were activated by EP with or without 150 μM BL I at various periods of maturation culture, and the nuclear status was evaluated.

According to the results of Experiments 1 and 2 and a previous report that a higher blastocyst rate was obtained when oocytes were matured in mTCM199 medium for 48 h and then activated by EP compared to oocytes cultured for 36 and 42 h [17], the oocytes matured for 48 h and activated by EP with 150 μM BL I were used in Experiment 3 and 4 to assess the effects of culture conditions on the developmental capacity of parthenogenetic embryos.

In Experiment 3, effect of the media for embryo culture on the rate of parthenogenetic development was examined. The activated oocytes (1 oocyte/μl) were cultured either (A) in modified Whitten's medium (mWM, with 94.8 mM NaCl) with 4 mg/ml BSA (Sigma) and 0.5 mg/ml hyaluronic acid (Sigma) for 48 h, and then in Whitten's medium (WM, same as mWM with NaCl decreased to 68.5 mM) for additional 5 days [26], or (B) in modified NCSU37 medium (mNCSU37) containing 4 mg/ml BSA (Sigma) and 5 μg/ml insulin [29] for 7 days at 38.5 C in an atmosphere of 5% CO_2 in air.

In Experiment 4, it was determined whether the oxygen concentration affects the developmental ability of parthenogenetic embryos. The activated oocytes were cultured according to the protocol (B) as described above for 7 days under two different oxygen environments (5% and 20%). The rates of embryos at the cleavage and blastocyst stages were evaluated at 48 h and 6–7 days after activation, respectively. The cell number of blastocysts was counted by using Hoechst 33342 epifluorescein staining.

Statistical analysis

We conducted statistical analysis of data using an analysis of variance (ANOVA) and a Fisher's protected least significant difference test along with the Stateview program software (Abacus Concepts, Berkeley, CA, USA). A probability of $P<0.05$ was set as the significance level.

Results

Experiment 1: as shown in Table 1, the rates of oocytes that underwent GVBD and matured to the M-II stage after culture 36, 42 and 48 h were not found to differ. Thus, most oocytes at 48 h of culture had been in the M-II stage for at least 12 h.

Experiment 2: as shown in Table 2, when oocytes were matured for 36, 42 and 48 h and activated by EP with or without BL I treatment, the rates of

Table 1. Effects of maturational age on their germinal vesicle break down (GVBD) and maturation to metaphase-II (M-II) of porcine oocytes matured *in vitro*

Maturational ages of oocytes (h)	No. of oocytes examined	No. of replicates	% of oocytes underwent GVBD	% of oocytes matured to M-II
36	49	3	95.7	79.6
42	49	3	100.0	81.6
48	49	3	100.0	83.6

Values are percentage of 3 independent experiments.

Table 2. Effects of maturational age and activation treatments on the nuclear status of porcine oocytes matured *in vitro* (mean ± SD)

Maturational ages and activation methods	No. of oocytes examined	%# of oocytes with 1PN1PB*	2PN1PB	abnormal nuclear status	%** of oocytes activated
36 h					
EP	74	51.8 ± 14.1[a]	19.4 ± 9.6[a]	6.9 ± 8.0[a]	63.0 ± 13.8[a]
EP + BL I	74	2.0 ± 1.2[b]	66.3 ± 10.1[b]	23.0 ± 16.3[b]	95.8 ± 3.3[b]
42 h					
EP	71	13.1 ± 10.3[b]	62.7 ± 10.1[b]	24.2 ± 15.5[b]	94.4 ± 4.5[b]
EP + BL I	75	2.0 ± 0.8[b]	74.1 ± 2.8[b]	18.6 ± 8.5[b]	97.1 ± 2.3[b]
48 h					
EP	69	10.9 ± 4.1[b]	63.9 ± 11.7[b]	25.2 ± 14.0[b]	96.3 ± 3.5[b]
EP + BL I	68	0	75.7 ± 7.8[b]	24.3 ± 7.8[b]	97.4 ± 2.1[b]

Values are percentages (mean ± SD) of 4 independent experiments.
[a-b] Within the same column, values with different superscripts differ ($P<0.05$).
*PN: pronucleus(ei); PB: polar body.
**% of oocytes activated = No. of oocytes activated/No. of oocytes examined.
#% of oocytes with each nuclear status = No. of oocytes with each nuclear status/No. of oocytes activated.

oocytes activated were not statistically different, except for that of oocytes cultured for 36 h and activated by EP without BL I treatment. Without BL I treatment, the rates of oocytes with abnormal nuclear and diploid (2PN1PB) status were significantly enhanced ($P<0.05$, respectively) for oocytes cultured for 42 h (24.2 ± 15.5; 62.7 ± 10.1) and 48 h (25.2 ± 14.0; 63.9 ± 11.7) compared to those cultured for 36 h (6.9 ± 8.0; 19.4 ± 9.6). In contrast, the rate of oocytes with haploid (1PN1PB) status was significantly decreased (13.1 ± 10.3; 10.9 ± 4.1 vs 51.8 ± 14.1; $P<0.05$). With BL I treatment, the nuclear development status of activated oocytes (1PN1PB, 2PN1PB and abnormal status) was significantly affected compared to those without BL I treatment in oocytes cultured for 36 h, but not in oocytes cultured for 42 and 48 h.

Experiment 3: as shown in Table 3, when cultured in mNCSU37 medium, the rates of embryos cleaved and developed to the blastocyst stage (at 6 and 7 days of activation) were significantly enhanced ($P<0.05$, respectively) than those cultured in mWM medium. However, the mean nuclear numbers of embryos in the two media were not significantly different.

Experiment 4: when activated oocytes were cultured for 6–7 days in mNCSU37 medium under 5% and 20% O_2, there were no differences in the rates of cleavage and blastocyst formation, and the mean nuclear number of a blastocyst (Table 4).

Discussion

In the present study a positive effect of the cyclin-dependent kinases (cdks) inhibitor BL I on the rates

Table 3. Effects of culture media on the development of porcine oocytes matured and activated *in vitro* (mean ± SD)

Media	No. of oocytes examined	% of cleaved (after 48 h of activation)	% of blastocysts*		No. of cells/ blastocyst
			6 days	7 days	
mWhitten	79	57.1 ± 14.9[a]	11.4 ± 6.3[a]	20.7 ± 7.7[a]	32.3 ± 11.5
mNCSU37	80	86.7 ± 6.3[b]	28.4 ± 9.7[b]	44.7 ± 10.7[b]	35.2 ± 10.0

Values are percentages (mean ± SD) of 4 independent experiments.
[a-b] Within the same column, values with different superscripts differ ($P<0.05$).
* Based on the examined oocytes.

Table 4. Effects of oxygen concentrations on the development of porcine oocytes matured and activated *in vitro* (mean ± SD)

Gas (O_2)	No. of oocytes examined	% of cleaved (after 48 h of activation)	% of blastocysts*		No. of cells/ blastocyst
			6 days	7 days	
5%	69	89.4 ± 6.2	29.3 ± 3.7	49.6 ± 15.0	44.1 ± 14.1
20%	63	88.2 ± 6.9	28.1 ± 11.8	45.9 ± 12.8	39.2±10.1

Values are percentages (mean ± SD) of 4 independent experiments.
*Based on the examined oocytes.

of activation of porcine oocytes matured *in vitro* and then treated with EP and CB was demonstrated, particularly on newly matured oocytes (36 h of maturation culture; Table 2). The probable reasons for this significantly lower rate of activation for oocytes matured after 36 h of culture might be that the immaturity of the metaphase spindle and inadequate cytoplasmic maturation of newly matured oocytes at the M-II stage caused re-activation of maturation promoting factor (MPF) after parthenogenetic activation by EP only [30]. The re-activation of MPF in the oocytes might be inhibited through addition of BL I to the medium, because BL I can induce a sharp decrease in MPF activity [23]. Moreover, oocytes cultured for 36 h showed a significantly lower rate of oocytes with two pronuclei after the activation treatment than those cultured for 42 and 48 h, suggesting that spindles had not yet fully developed in newly matured oocytes. Therefore, it can be concluded that the complete nuclear and cytoplasmic maturation of oocytes needs an additional culture after reaching the M-II stage. Similar results were obtained by using a combination of EP and protein synthesis inhibitor, cycloheximide (CHX), in pigs [16], cattle [31].

BL I, which acts as a competitive inhibitor of ATP, is a potent and specific inhibitor of cyclin-dependent kinases (cdks) and has few inhibitory effects on other protein kinases such as MAP kinases [32, 33]. Kubelka *et al.* (2002) showed that the timing of exposure of oocytes to BL I impaired the time course of cdc2 and MAP kinase activities, cdc2 kinase became inactivated after 3 h of BL I exposure and MAP kinase became inactivated after about 5 h and 8 h of BL I treatment in cattle and pig oocytes. It was suggested that the inactivation of MAP kinase might not be induced by BL I but triggered by the inactivation of cdc2 kinase. Thus only cdc2 kinase might be inactivated in the activated oocytes exposed to BL I for 4 h in the present study. However, the high rates (45–50%) of oocytes matured in vitro for 48 h developed to the blastocyst stage at 7 days of culture after activation (Table 3, 4). The reasons for these high rates might be that BL I only inhibited cdc2 kinase, not other kinases or proteins, which might be needed for a further developmental capacity of activated oocytes. In contrast, only 12–36% of oocytes did so after 7 days of culture when the oocytes were matured for 32–48 h and activated by a combination of EP and CHX [16], and 11–38% of oocytes matured in modified TCM 199 and NCSU23 for 36–48 h and activated by a combination of EP and CHX developed to the blastocyst stage after 7 days of culture in our recent

study (unpublished). Although effects of CHX and BL I have not been compared directly in the present study, CHX may nonspecifically influence a number of proteins and protein kinase activities, as well as cdc2/cyclin B kinases resulting in impaired developmental capacity of the parthenotes.

Treatment with CB, a protein synthesis inhibitor, after parthenogenetic activation of porcine oocytes has been significantly shown to suppress the extrusion of the second polar body, and enhance their developmental capacity to the blastocyst stage [13, 34]. Presumptive porcine diploid oocytes produced by CB treatment just after EP showed a relatively high developmental ability to the blastocyst stage [27, 28]. The similar results were obtained in mice [35, 36]. When the duration of maturation culture of oocytes was prolonged to 48 h in the present study, an increasing tendency on the rate of oocytes with two pronuclei (2PN1PB) after activation by EP with BL I was shown. Based on this result and our previous report [17], it was decided to culture oocytes for 48 h to evaluate that effects of IVC conditions on the subsequent development of activated oocytes.

The ability of two IVC media, modified Whitten's and NCSU37 media, to support the development of parthenogenetic embryos to the blastocyst stage was examined. Increasing the osmotic concentration through changing the ratio of Na^+/K^+ in WM for the first 2 days of culture was reported to improve cleavage and blastocysts rates [26, 37, 38]. Addition of hyaluronic acid to WM was also proven to be beneficial for the development of porcine parthenogenetic embryos [34]. Therefore the culture system with modified WM established by Kurebayashi *et al.* (1995) (mWM system) was used in the present study. Another culture system based on NCSU37 medium supplemented with insulin [29] (mNCSU37 system) was compared with the mWM system. Although the mean cell numbers of a blastocyst were not different between the two systems, significantly higher rates of cleavage and blastocyst formation were obtained for the mNCSU37 system. These results indicated that the mNCSU37 system was more appropriate for the development of porcine parthenogenetic embryos than the mWM system. Recently high blastocyst yields were also produced when oocytes matured and fertilized *in vitro* were cultured in mNCSU37 medium, and 19 piglets were born from 3 pregnant recipients after transfer of in vitro produced blastocysts [39]. Therefore, it is suggested that mNCSU37 medium might be a more suitable for IVC of porcine embryos to produce either IVF or parthenogenetic activation.

It is generally accepted that a low oxygen tension environment has a beneficial effect on early embryo development in a variety of species [mice, (40); rabbit, (41); pig, (42); sheep, (43); cattle, (43, 44)]. However, some results are also contradictory. In the pig, the precise effects of oxygen concentrations during IVC on the development of porcine embryos were not well understood. The blastocyst yields had not been affected [45, 46] by reduction of O_2 tension during IVC, but cell numbers per a blastocyst were increased [47] or decreased [45] in *in vivo* produced porcine embryos. Callesen *et al.* (2000) reported that a significantly higher blastocyst yield was achieved when parthenogenetic embryos produced *in vitro* were cultured under a low oxygen tension (5%)[46]. However, in the present study, neither blastocyst yields nor the mean cell number per a blastocyst were affected by reduction of O_2 tension during IVC. The explanations of these different results might be based on 1) the abilities of the different types of embryos to tolerate oxygen radicals, 2) the different conditions used for culture and activation, 3) the maturity of different sources of oocytes. Although the mean cell numbers of a blastocyst were not statistically different between parthenotes cultured in two gaseous atmospheres in the present study, more frequencies for blastocysts having over 50–60 cells were observed in 5% O_2 tension. A low O_2 environment during IVM of porcine oocytes also promotes the cell number of embryos produced *in vitro* [39]. Taken together, it is postulated that a low O_2 concentration can be considered to improve the quality of embryos through a reduction of oxidative stress.

Our results demonstrated that the combined electric pulse and BL I treatment of porcine IVM oocytes is capable of producing high rates of good quality blastocysts when cultured in a suitable *in vitro* condition.

Acknowledgements

The authors wish to thank K. Kawamura and N. Nishimoto for their excellent support in the experiments. This study was supported by the

Japan Science and Technology Corporation (JST) and a Grant-in-Aid (Bio Design Program) from the Ministry of Agriculture, Forestry and Fisheries (BDP-03-III-1).

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