Effects of Butyrolactone I on the Inhibition of Meiotic Resumption of Porcine Oocytes and Subsequent Development after Somatic Cell Nuclear Transfer

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Abstract: *We examined the effects of butyrolactone I (BL-I), a specific cdc2 kinase inhibitor, on the inhibition of meiotic resumption of porcine oocytes and subsequent developmental competence after somatic cell nuclear transfer (NT). Porcine follicular oocytes were cultured in a medium containing BL-I during maturation culture. BL-I suppressed germinal vesicle (GV) breakdown in a dose-dependent manner and most (98.3%) oocytes were arrested at the GV stage when they were cultured for 28 h with 100 μM BL-I, but after 48 h of culture with 100 μM BL-I half of the oocytes underwent GV breakdown. Twenty hours after release from the BL-I treatment (28-h culture), most (95.9%) oocytes reached the metaphase II stage, whereas most non-treated oocytes reached the metaphase II stage after 40 h of maturation culture. There were no differences between BL-I-treated and non-treated oocytes in meiotic progression. When oocytes were enucleated and fused with serum-starved cumulus cells, the development of NT embryos to the blastocyst stage with the BL-I-treated oocytes (12.3%) was similar to that achieved with non-treated oocytes (12.5%). The results demonstrate that BL-I could reversibly inhibit meiotic resumption of porcine oocytes, and that BL-I-treated porcine oocytes can be used as recipient cytoplasts for NT without compromising developmental competence.*

Key words: *Butyrolactone I, Nuclear transfer, Oocyte, Pig, Maturation culture*

The production of cloned offspring by somatic cell nuclear transfer (NT) has been achieved in many species including pigs [1–3], but the *in vitro* development of porcine NT embryos constructed with *in vitro* matured oocytes is still limited. Sub-optimal *in vitro* maturation culture conditions might be one of the causes of the low developmental competence of porcine oocytes, and an improvement in *in vitro* maturation culture conditions is needed.

Mammalian oocytes are arrested at the G_2/M phase transition of the first meiotic division and spontaneously resume meiosis when they are liberated from follicles. Inhibition of spontaneous meiotic resumption during the culture for oocyte maturation has been shown to improve the maturational and developmental competence of growing [4–7] and fully grown [8, 9] oocytes in domestic species. The oocytes accumulate mRNA and some proteins which are essential for the acquisition of maturational and developmental competence while meiotic resumption is being inhibited [6, 10]. Butyrolactone I (BL-I) and roscovitine are specific cdc2 kinase inhibitors which compete with ATP [11] and inhibit meiotic resumption of bovine [6, 9, 12, 13] and porcine [10, 14–16] oocytes. Porcine oocytes could be arrested at the germinal vesicle (GV) stage for up to 28 h in a protein-free medium supplemented with BL-I at a concentration of 12.5 to 20 μM [14, 15], but BL-I treatment for 20 h in a protein-free medium did not improve the developmental competence of porcine oocytes after *in vitro* fertilization [15]. It is likely that a 20-h-treatment with BL-I in a protein-free medium is not long enough for the acquisition of developmental competence in porcine oocytes. The addition of porcine follicular fluid (pFF) to the maturation medium with BL-I might improve the cytoplasmic maturation of porcine oocytes, because pFF added to the maturation medium enhanced the *in vitro* maturation and subsequent

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fertilization of porcine oocytes [17–19]. Nevertheless, the effect of the period of BL-I treatment in the presence of pFF on the inhibition of meiotic resumption of porcine oocytes has not been reported. Furthermore, the meiotic progression and subsequent developmental competence of porcine oocytes treated with BL-I for more than 20 h has not been examined.

This study examined the optimal concentration of BL-I in the presence of pFF and culture period for GV breakdown of porcine oocytes. We also examined the reversibility and subsequent meiotic progression of BL-I treatment. Finally, the development of NT embryos constructed with BL-I-treated oocytes was compared with that of embryos produced with non-treated oocytes to examine the effect of BL-I treatment on the developmental competence of oocytes at the cytoplasmic level.

Materials and Methods

Oocyte collection and maturation in vitro

Oocyte-cumulus-granulosa cell complexes (OCGCs) were recovered from abattoir-derived gilt ovaries after dissecting healthy antral follicles (4 to 8 mm in diameter) and scraping the inner surface of the follicles with a pair of forceps in TCM-199 (Cat No. M-5017, Sigma Co., St. Louis, MO, USA) supplemented with 25 mM HEPES (Sigma) and 0.1% polyvinyl alcohol (Sigma). *In vitro* maturation culture was performed with a maturation medium: bovine serum albumin (BSA)-free NCSU-23 [20] supplemented with 10% pFF, 10 ng/ml of human recombinant epidermal growth factor (Sigma), 0.57 mM cysteine (Sigma), 10 IU/ml of equine chorionic gonadotropin (Serotropin, Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan) and 10 IU/ml of human chorionic gonadotropin (Gonatropin, Teikoku Hormone). The pFF collected from prepubertal pig ovarian follicles 4 to 6 mm in diameter was filtered twice through 0.45-μm filters and stored at –40°C until use. OCGCs were cultured in the maturation medium at 39°C under a humidified atmosphere of 5% CO_2 in air. After maturation culture, the oocytes were freed from cumulus cells by repeated pipetting and vortexing in Dulbecco's phosphate-buffered saline (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 0.1% hyaluronidase (Sigma).

Evaluation of the meiotic stage of oocytes

After maturation culture, oocytes were mounted on a glass slide and fixed with an acetic acid and ethanol mixture (1:3) for at least 48 h. After staining with 1% aceto-orcein, the oocyte meiotic stage was evaluated under a phase-contrast microscope.

Preparation and in vitro culture of NT embryos

NT was performed as described previously [21, 22] with a slight modification. In brief, cumulus cells isolated from OCGCs after maturation culture were cultured for 3 or 4 days in Dulbecco's modified Eagle's medium: Nutrient mixture F12 (DMEM/F12, Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, Gibco) in a 4-well culture plate (Nalge Nunclon International, Roskilde, Denmark). They were further cultured in DMEM/F12 supplemented with 0.5% FCS for 3 to 5 days. Enucleated oocytes and medium-sized cumulus cells (20 to 24 μm in diameter), of which 95.3% were in the G_0/G_1 phase, were subjected to a single DC pulse of 150 V/mm for 100 μsec from an Electro Cell Fusion (LF-100 Life Tech Co., Tokyo, Japan) to induce fusion and activation simultaneously. Fused couplets were cultured for 6 h in NCSU-23 supplemented with 10 μg/ml of cycloheximide (Sigma). Subsequent *in vitro* development of NT embryos was examined by culturing for 7 days in NCSU-23 under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39°C. Cleavage and development to blastocysts were recorded on Days 2 and 7 (Day 0 = day of activation), respectively. The number of blastocyst cells was assessed on Day 7 of culture by an air-dry method [23].

Experimental design

Experiment 1: OCGCs were cultured for 28 h in maturation medium containing various concentrations (0–100 μM) of BL-I (Funakoshi Co., Ltd., Tokyo, Japan) to examine the effect of the concentration of BL-I on the inhibition of oocyte meiotic resumption.

Experiment 2: OCGCs were cultured for 0-72 h in maturation medium containing 100 μM BL-I to examine the effect of the period of culture with BL-I on the inhibition of oocyte meiotic resumption.

Experiment 3: To confirm the reversibility of the oocyte meiotic inhibition with BL-I, the meiotic progression of BL-I-treated oocytes was compared with that of non-treated oocytes (controls). OCGCs were cultured for 28 h in the maturation medium with 100 μM BL-I and were further cultured for 4 to 24 h without BL-I. For the controls, OCGCs were cultured for 20 h in the maturation medium without supplementation with BL-I and another 4 to 24 h without hormones [24].

Experiment 4: To examine the developmental competence of the oocytes treated with BL-I, we

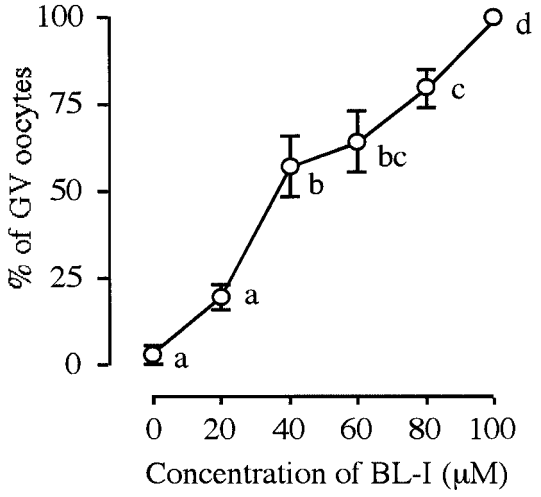

Fig. 1. Effect of butyrolactone I (BL-I) concentration on the meiotic resumption of porcine oocytes cultured for 28 h. Data are means ± SEM of 3–6 replicates (total numbers of oocytes in each group were 46 to 85). [a, b, c, d] Different superscripts denote significant differences ($P<0.01$).

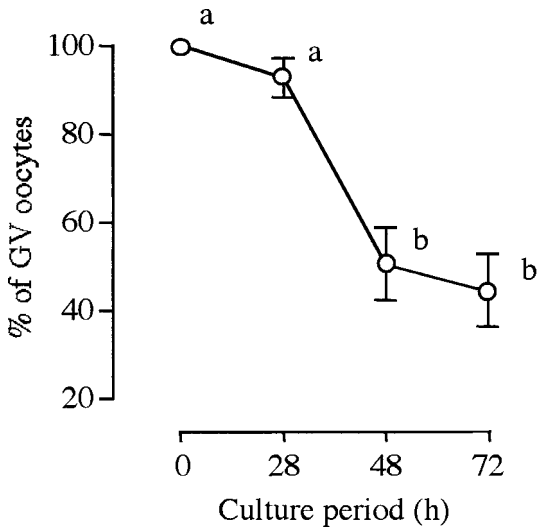

Fig. 2. Effect of culture period on the meiotic resumption of porcine oocytes in the presence of 100 μM butyrolactone I. Data are means ± SEM of 3-5 replicates (total numbers of oocytes in each group were 46 to 85). [a, b] Different superscripts denote a significant difference ($P<0.05$).

compared the *in vitro* development of porcine NT embryos constructed with BL-I-treated and non-treated oocytes. One group of oocytes were cultured for 28 h in maturation medium containing 100 μM BL-I and for an additional 20 h without BL-I. The control group of oocytes were cultured for 20 h in the maturation medium without supplementation with BL-I and for an additional 20 h without hormones [24].

Statistical analysis

Data were subjected to one-way ANOVA followed by Fisher's protected least significant difference test (Experiments 1 to 3) or Student's *t*-test (Experiment 4) with StatView software (Abacus Conceptus Inc., Berkeley, CA, USA).

Results

Experiment 1: BL-I suppressed GV breakdown in a dose-dependent manner, and most (98.3 ± 2.9%) oocytes were arrested at the GV stage in the presence of 100 μM BL-I (Fig. 1).

Experiment 2: Although BL-I inhibited GV breakdown until 28 h of culture, the proportion of oocytes arrested at the GV stage decreased ($P<0.05$) after 48 h of culture (Fig. 2).

Experiment 3: Without BL-I treatment, most oocytes showed signs of GV breakdown by 28 h of culture (97.9 ± 3.6%, mean ± SD of 3 replicates) and reached the MII stage after 40 h of maturation culture (97.8 ± 3.8%, mean ± SD of 3 replicates, Fig. 3). When the oocytes were treated with BL-I for 28 h, most of them showed signs of GV breakdown at 36 h after the start of culture (97.8 ± 3.8%, mean ± SD of 3 replicates) and reached the MII stage by 48 h (95.9 ± 3.6%, mean ± SD of 3 replicates). The interval between GV breakdown and MI, and between MI and MII in the BL-I-treated oocytes was similar to that in non-treated oocytes.

Experiment 4: The enucleation rate in BL-I-treated oocytes (62.7 ± 6.5%, mean ± SD of 4 replicates) was similar to that in the non-treated oocytes (68.6 ± 9.1%, mean ± SD of 4 replicates, $P>0.1$). As shown in Table 1, the development of NT embryos constructed with BL-I-treated treated and non-treated oocytes did not differ in terms of cleavage, development to the blastocyst stage or the number of blastocyst cells ($P>0.1$).

Discussion

BL-I was reported to maintain porcine oocytes at the GV stage for up to 28 h in a protein-free medium at a concentration of 12.5 to 20 μM [14, 15]; whereas 100 μM of BL-I was needed to prevent meiotic resumption

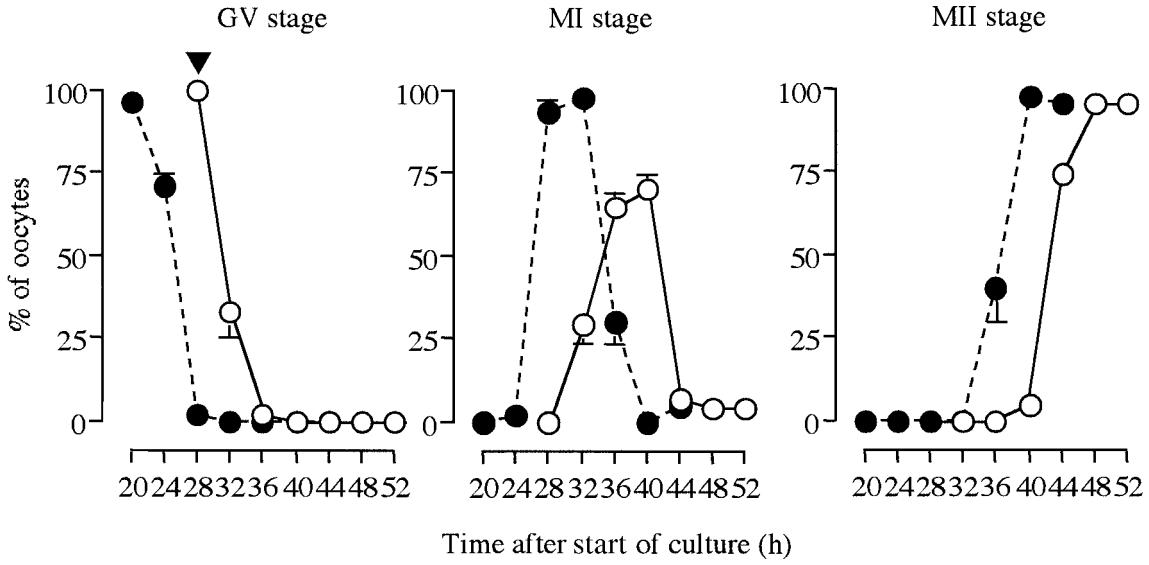

Fig. 3. Meiotic progression of porcine oocytes treated with (—○—) or without (– –●– –) butyrolactone I (BL-I). For the BL-I treatment, oocytes were cultured for 28 h in the maturation medium with 100 μM of BL-I and for 4–24 h in the maturation medium without BL-I. The arrowhead represents the timing of release from treatment with 100 μM BL-I. Control oocytes were cultured for 20 h in the maturation medium and for an additional 4–24 h in the maturation medium without hormones. Data are means ± SEM of 3-5 replicates (total numbers of oocytes in each group were 46 to 72).

Table 1. Development of nuclear transfer embryos constructed with butyrolactone I (BL-I) treated or non-treated porcine oocytes

Item	BL-I treatment	
	Without [a]	With [b]
No. of couplets in attempted fusion	82	75
% of couplets fused	84.7 ± 13.3	85.8 ± 9.1
% of ≥2-cell on day 2 [c]	61.7 ± 10.9	63.3 ± 3.8
% of blastocysts on day 7 [c]	12.3 ± 11.7	12.5 ± 6.7
Blastocyst Cell No. (n)	30.4 ± 9.8 (9)	28.1 ± 10.6 (8)

% values are means ± SD of 4 replicates. [a] Oocytes were cultured for 28 h in the maturation medium with 100 μM of BL-I and for 20 h without BL-I. [b] Oocytes were cultured for 20 h in the maturation medium and for an additional 20 h without hormones. [c] Based on the number fused.

for the same period under the present conditions. Half of the present porcine oocytes underwent GV breakdown after 48 h of culture in the presence of 100 μM BL-I. Although the effect of prolonged BL-I treatment for more than 28 h in a protein-free medium on the inhibition of porcine oocyte meiotic resumption has not been examined, meiotic arrest of bovine oocytes could be achieved with 100 μM BL-I for up to 48 h in protein-free or BSA-supplemented media [25, 26]. In the present study, OCGCs were cultured in the presence of pFF. The necessity for a higher dose of BL-I and shorter period for the inhibition of porcine oocyte meiotic resumption in the present study might be due to the binding of BL-I to the component(s) of pFF added to the maturation culture medium. The addition of protein (serum or BSA) to the culture medium reduced the inhibitory effect of BL-I on the meiotic resumption of bovine oocytes [25]. Although 100 μM BL-I was required to inhibit the meiotic resumption of porcine oocytes for 28 h, prolonged inhibition in the presence of proteins might be required at more than 100 μM BL-I.

After release from 28-h BL-I treatment, the BL-I-treated oocytes showed similar meiotic progression to the non-treated oocytes (Fig. 3), suggesting that BL-I treatment has no adverse effect on the meiotic resumption and subsequent meiotic progression of porcine oocytes. The presence of gonadotropins during maturation culture may affect the meiotic progression and maturation of porcine oocytes: the removal of gonadotropins after 20 h of *in vitro* maturation culture (around the time of GV breakdown) enhanced the cytoplasmic maturation of porcine oocytes [24]. In the present study the BL-I-treated oocytes were cultured in maturation medium supplemented with gonadotropins throughout the maturation culture period even after the release from the BL-I treatment. Further experiments are therefore needed to determine the requirement of gonadotropins after release from the BL-I treatment.

Most of the BL-I-treated and non-treated oocytes required 48 h and 40 h of maturation culture, respectively, to reach the MII stage (Fig. 3). Prolonged maturation culture might lead to aging and reduce the developmental competence of oocytes in cattle [27] and pigs [21, 22]. We therefore used the BL-I-treated and non-treated (control) oocytes as recipient cytoplasts after 48 and 40 h of maturation culture, respectively. Neither a detrimental nor a beneficial effect of BL-I treatment on oocyte developmental competence was observed after the use of BL-I-treated oocytes as a recipient cytoplast for NT. The result was consistent with a previous finding that the development of *in vitro* fertilized porcine oocytes treated with BL-I in a protein-free medium for 20 h prior to 24 h of maturation culture was similar to that of non-treated oocytes which were cultured for 44 h [15].

The time schedules of porcine *in vitro* fertilization and NT procedures could be altered by the BL-I treatment since there was no harmful effect of BL-I treatment for 28 h on maturational or developmental competence. BL-I treatment will also make the pre-culture of porcine oocytes possible. The pre-culture of bovine and porcine oocytes in a protein-free medium supplemented with BL-I had neither a beneficial nor a detrimental effect on their developmental competence [12, 15]. It was, however, indicated that the pre-culture of bovine oocytes in the presence of FCS and BL-I under a low (5%) oxygen condition before induction of oocyte meiotic resumption could improve developmental competence after fertilization *in vitro* [9]. More experiments are needed to clarify the effect of pre-culture with BL-I in maturation media supplemented with or without proteins such as FCS on the developmental competence of porcine oocytes.

The results demonstrated that meiotic resumption of porcine oocytes could be reversibly inhibited for 28 h in a medium containing 100 μM BL-I and pFF, and that BL-I-treated porcine oocytes can be used as recipient cytoplast for NT without compromising developmental competence.

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