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Meiotic inhibition with different cyclin-dependent kinase inhibitors in bovine oocytes and its effects on maturation and embryo development

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Summary

Cyclin-dependent kinase inhibitors (CDKIs) such as butyrolactone I (BL-I) and roscovitine (ROS) maintain bovine oocytes blocked at the germinal vesicle (GV) stage. Bohemine (BOH), another CDKI, has been used for oocyte activation. The objective of this study was to determine whether BOH blocks meiosis and to compare its efficiency with other CDKIs (ROS and BL-I). Oocytes were cultured for 24 h in 0, 50, 100 and 150 μM BOH to determine the best concentration for blocking meiosis (experiment 1). GV rates were 3.3%, 64.5%, 83.3% and 88.9% (0, 50, 100 and 150 μM, respectively). Experiment 2 compared meiotic inhibition efficiency of BOH (100 μM), ROS (25 μM) and BL-I (100 μM). BL-I presented the highest GV rates (97.5%). BOH and ROS were similar to each other (85.4% and 79.9%, respectively). To assess the reversibility of meiotic inhibition (experiment 3), oocytes underwent *in vitro* maturation (IVM) for 18 h after the 24 h inhibition. Control oocytes were submitted to IVM for 18 h (C18) or 24 h (C24). Maturation rates were either similar to (ROS and BL-I: 96.0% and 93.6%, respectively) or superior to (BOH, 96.9%) C24 (91.0%). All groups were superior to C18 (82.5%). In experiment 4, oocytes were treated as in experiment 3 and then *in vitro* fertilized and cultured for 8 days. Blastocyst rates for BL-I (32.3%) were similar to C24 (35.0%), while those for BOH (20.2%) and ROS (24.2%) were inferior. All groups were inferior to C18 (43.4%). The results show that: (a) BOH inhibits meiosis resumption; (b) BL-I is the most effective of the CDKIs tested for blocking meiosis; (c) culture of oocytes with meiosis inhibitors is fully reversible in terms of nuclear maturation but they may either decrease (BOH and ROS) or maintain (BL-I) embryo development rates.

Keywords: Bohemine, Bovine oocytes, Butyrolactone I, Meiotic inhibition, Roscovitine

Introduction

In mammals oogenesis begins during fetal life and oocytes remain in prophase I until shortly before ovulation, when they resume meiosis (oocyte maturation). Meiosis resumption is initiated by the activation of maturation promoting factor (MPF) leading the oocyte to progress from prophase I to metaphase II (MII). During MII, MPF remains at high levels, maintaining the oocyte held at this stage until fertilization or parthenogenetic activation (Motlik *et al*., 1998).

It has been suggested that before meiosis resumption *in vivo*, oocytes undergo a process termed capacitation (Hyttel *et al*., 1997; Sirard, 2001). This process occurs towards the end of oocyte growth before meiosis resumption and involves several changes at the ultrastructural level (Hyttel *et al*., 1997). These changes may be related to the acquisition of developmental competence by the oocytes. According to Blondin *et al*. (1997) developmental competence is acquired before the actual maturation period.

In vitro maturation procedures use oocytes removed from 2–6 mm follicles; once removed from the inhibiting environment of the follicle, the oocyted

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immediately resume meiosis. This drives the oocytes not to undergo the capacitation period and may have a bearing on the lower developmental competence of the oocytes when compared with *in vivo* matured oocytes (Hyttel *et al*., 1997). Recently, Rizos *et al*. (2002) have demonstrated that the main factor affecting blastocyst rates is the intrinsic quality of the oocyte, while *in vitro* culture influences the quality of developing blastocysts. This intrinsic oocyte quality can be related to the capacitation period observed *in vivo*.

It is believed that developmental competence may be improved if oocytes are maintained in meiotic arrest for a period (pre-maturation culture) before they resume meiosis, in an effort to mimic capacitation (Hyttel *et al*., 1997; Lonergan *et al*., 2000). Studies have demonstrated meiotic inhibition in mammalian oocytes by using either a protein synthesis inhibitor or phosphorylation inhibitors (cycloheximide: Saeki *et al*., 1997; 6-DMAP: Saeki *et al*., 1997; Avery *et al*., 1998; Dode & Adona, 2001). However, these drugs were shown to decrease the developmental potential of treated oocytes, probably because they are non-specific and could be inhibiting pathways necessary for competence. However, cyclin-dependent kinase inhibitors (CDKIs) act specifically on MPF (Kubelka *et al*. 2000) and could, therefore, be more effective.

Bovine oocytes have been successfully blocked at the germinal vesicle (GV) stage using CDKIs such as butyrolactone I (Kubelka *et al*., 2000; Hashimoto *et al*., 2002) and roscovitine (Mermillod *et al*., 2000) by specifically blocking MPF activity. Bohemine is another synthetic CDKI (Mad'arova *et al*., 2002) and has been used for oocyte activation (Alberio *et al*., 2000) after intracytoplasmic sperm injection and nuclear transfer (Motlik *et al*., 1999; Alberio *et al*., 2001). There are no data, however, on its use for meiotic inhibition.

The present study aimed to determine whether bohemine blocks meiosis and to compare its effectiveness with other CDKIs (butyrolactone I and roscovitine) regarding meiotic inhibition and subsequent nuclear *in vitro* maturation in bovine oocytes, as well as its effects on embryo development after *in vitro* fertilization and culture.

Materials and methods

Chemicals were purchased from Sigma Chemical (St Louis, MO) unless otherwise indicated. Inhibitors were prepared, separately, as a stock solution of 50 mM in dimethylsulphoxide (DMSO), aliquoted and stored at −20 °C until use. Butyrolactone-I (BL-I) was purchased from Biomol (Plymouth Meeting, PA), roscovitine (ROS) from Sigma and bohemine (BOH) was a kind gift from Dr Mirek Strnad (Palacký University, Czech Republic).

Oocyte collection and culture

Bovine ovaries were collected at a local abattoir and brought back to the laboratory soon after slaughter in sterile saline (0.9% NaCl) supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin sulphate) at 30 °C. Oocytes were aspirated from 2–6 mm follicles using a 19-gauge needle connected to a vacuum pump (90 mmHg). Recovered oocytes with homogeneous cytoplasm and at least three layers of cumulus cells were washed three times in TCM-199 supplemented with antibiotics and 10% fetal calf serum (FCS; Gibco, Grand Island, NY) and then cultured for meiotic inhibition or *in vitro* maturation (IVM).

For meiotic inhibition, oocytes were cultured for 24 h in TCM-199 supplemented with 3 mg/ml bovine serum albumin (BSA) (TCM-BSA) and one of the inhibitors (BOH, ROS or BL I). BOH was used at 50, 100 and 150 μM for the first experiment. For the other experiments BOH was used at 100 μM, ROS at 25 μM (Mermillod *et al*., 2000) and BL-I at 100 μM (Kubelka *et al*., 2000).

For IVM (reversion of meiotic inhibition), oocytes were cultured in TCM-199 supplemented 10% FCS, 0.5 μg/ml FSH, 5.0 μg/ml LH and antibiotics for 18 or 24 h. All cultures (meiotic inhibition, IVM, fertilization and culture) were in 100 μl droplets (20–30 oocytes per droplet) of the appropriate medium under mineral oil, at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity.

Fixation, staining and morphological analysis

To determine stage of meiosis, oocytes were stripped from their cumulus cells by vortexing for 3 min in 0.3 ml saline solution + 1% FCS in a 2.5 ml tube and then placed between a slide and a coverslip. Fixation was in ethanol:acetic acid (3:1, Merck, Rio de Janeiro, Brazil) for 24 h and staining in 2% acetic orcein. Oocytes were observed under a phase-contrast microscope and classified as: germinal vesicle stage (GV, immature oocytes), metaphase I (MI), anaphase I (AI), telophase I (TI) or metaphase II (MII, matured oocytes). Oocytes remaining at the GV stage after inhibition were considered to be completely blocked and oocytes in MII after IVM were considered to have fully reversed the meiotic inhibition. Oocytes reaching MI, AI and TI were considered as intermediate stages.

In vitro fertilization

For *in vitro* fertilization (IVF), frozen thawed semen from the same bull (Genética Avançada, São Carlos, Brazil) was prepared by Percoll (Pharmacia, Uppsala, Sweden) gradient technique. Two millilitres of 45% Percoll were added over 2 ml of 90% Percoll and thawed semen was added and centrifuged for 20 min

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Table 1 Meiotic inhibition in bovine oocytes cultured *in vitro* in the presence of different concentrations of bohemine for 24 h

		Stage of meiosis		
Bohemine	Oocytes No. (%)	GV No. (%)	Intermediate No. (%)	MII No. (%)
0 μM	103	3 (3.3 ± 3.7)[a]	9 (8.4 ± 3.2)[a]	91 (88.3 ± 2.4)[a]
50 μM	94	60 (64.5 ± 8.7)[b]	1 (1.0 ± 1.8)[a]	33 (34.4 ± 6.9)[b]
100 μM	104	86 (83.3 ± 2.5)[c]	0 (0 ± 0)[a]	18 (16.7 ± 2.5)[c]
150 μM	105	93 (88.9 ± 3.6)[c]	0 (0 ± 0)[a]	12(11.1 ± 3.7)[c]

Data are presented as the mean ± SEM of four replicates.
GV, germinal vesicle. Intermediate stages comprise: MI, metaphase I; AI, anaphase I; TI, telophase I. MII, metaphase II.
[a–c] Different letters within the same column indicate significant difference between treatments ($p < 0.05$).

at 700 *g*. The supernatant was then removed and 5 ml TALP (Parrish *et al*., 1988) was added to the pellet and centrifuged again for another 2 min at 200 *g*. Separated motile spermatozoa were added to the fertilization droplet at a final concentration of 2×10^6 sperm cells/ml. IVF medium was TALP supplemented with 2 μM penicillamine, 1 μM hypotaurine, 250 μM epinephrine and 20 μg/ml heparin. Oocytes and sperm were co-incubated for 18 h under the same temperature and atmosphere conditions used for IVM.

In vitro culture

After the 18 h sperm–oocyte co-culture, presumptive zygotes were washed and transferred to the *in vitro* culture (IVC) medium (TCM 199 + 10% FCS and antibiotics). Forty-eight hours after insemination, cumulus cells were removed by pipetting and cleavage rates recorded. Only cleaved oocytes were maintained in the droplet in co-culture with their own removed cumulus cells. Blastocyst development rates were recorded at day 8 of IVC. The temperature and gas atmosphere were the same as used for IVM and IVF.

Experimental design

Experiment 1: Efficiency of different concentrations of bohemine in inducing meiotic inhibition
Oocytes were cultured for 24 h with increasing concentrations of BOH (50, 100 and 150 μM BOH). As control, one group was cultured in the same medium (TCM-BSA) in the absence of BOH. An additional group was fixed immediately after removal from follicles. At the end of culture, oocytes were fixed and stained to determine the stage of meiosis. Those oocytes remaining in GV stage were considered to be blocked.

Experiment 2: Meiotic inhibition with different inhibitors
Oocytes were cultured for 24 h in TCM-BSA, but supplemented with 100 μM BOH, 25 μM ROS or 100 μM BL-I, and assessed for meiotic stage as previously described.

Experiment 3: Reversibility of meiotic inhibition using different inhibitors
Oocytes were cultured for 24 h in inhibiting media (BOH, ROS or BL-I) followed by IVM for 18 h. Control oocytes were submitted to IVM for 18 h (C18) or 24 h (C24). By the end of culture, oocytes were evaluated for nuclear maturation stage. Those oocytes in MII were considered to have undergone reversion of the meiotic inhibition.

Experiment 4: Effect of meiotic inhibition using different inhibitors on embryo development
Oocytes were cultured in the same groups as described for experiment 3. After IVM, all groups were submitted to IVF and IVC, and cleavage and blastocyst rates were recorded.

Statistical analysis

For comparisons of maturation, inhibition, cleavage and blastocyst rates between treatments, data (4–6 replicates in each experiment) were analysed by ANOVA and Duncan test at a 5% level of significance. All data in percentages were arcsin-transformed prior to ANOVA.

Results

Experiment 1

In order to assess meiotic inhibition with BOH, the first step was to determine the dose–effect response to the inhibitor. Results are shown in Table 1. BOH was shown to be effective for blocking oocytes at the GV stage at all concentrations studied, with oocytes in GV stage in 64.5 ± 8.7%, 83.3 ± 2.5% and 88.9 ± 3.6% of cases for the concentrations of 50, 100 and 150 μM, respectively. In the control group (0 μM), only 3.3 ± 3.7% of the oocytes were at the GV stage, which was significantly different from the treated groups ($p < 0.05$). However, the higher concentrations of BOH

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Table 2 Meiotic inhibition in bovine oocytes cultured *in vitro* with different CDKIs for 24 h

Treatment	Oocytes No.	Stage of meiosis		
		GV No. (%)	Intermediate No. (%)	MII No. (%)
Control	110	21 (18.7 ± 7.7)[a]	3 (2.8 ± 3.3)[a]	86 (78.5 ± 7.2)[a]
BOH 100 μM	116	99 (85.4 ± 1.9)[b]	0 (0 ± 0)[a]	17 (14.6 ± 1.9)[b]
ROS 25 μM	119	95 (79.9 ± 2.1)[b]	3 (2.6 ± 3.1)[a]	21(17.5 ± 3.9)[b]
BL-I 100 μM	115	112 (97.5 ± 3.0)[c]	3 (2.5 ± 3.0)[a]	0 (0 ± 0.0)[c]

Data are presented as the mean ± SEM of five replicates.
GV, germinal vesicle stage. Intermediate stages comprise: MI, metaphase I; AI, anaphase I; TI, telophase I. MII, metaphase II.
BOH, bohemine; ROS, roscovitine; BL-I, butyrolactone-I.
[a−c] Different letters within the same column indicate significant difference between treatments ($p < 0.05$).

Table 3 Reversibility of meiotic inhibition in bovine oocytes treated *in vitro* with different CDKIs for 24 h followed by *in vitro* maturation for 18 h

Treatment	Oocytes No.	Stage of meiosis		
		GV No. (%)	Intermediate No. (%)	MII No. (%)
Control – 18 h	97	1 (1.1 ± 1.7)[a]	16 (16.4 ± 1.9)[a]	80 (82.5 ± 1.2)[a]
Control – 24 h	88	1 (1.1 ± 1.6)[a]	7 (8.0 ± 1.5)[b]	80 (91.0 ± 2.5)[b]
BOH 100 μM	96	0 (0 ± 0)[a]	3 (3.1 ± 3.1)[b]	93 (96.9 ± 3.1)[c]
ROS 25 μM	99	0 (0 ± 0)[a]	4 (4.1 ± 2.1)[b]	95 (96.0 ± 2.1)[bc]
BL-I 100 μM	92	0 (0 ± 0)[a]	6 (6.5 ± 3.0)[b]	86 (93.6 ± 3.0)[bc]

Data are presented as the mean ± SEM of four replicates.
GV, germinal vesicle stage. Intermediate stages comprise: MI, metaphase I; AI, anaphase I; TI, telophase I. MII, metaphase II.
Control – 18 h, 18 h IVM; control – 24 h, 24 h IVM; BOH, bohemine; ROS, roscovitine; BL-I, butyrolactone-I; bohemine.
[a−c] Different letters within the same column indicate significant difference between treatments ($p < 0.05$).

(100 and 150 μM) were significantly more effective ($p < 0.05$) than 50 μM. Intermediate-stage oocytes were only observed in the 0 and 50 μM concentrations (8.4 ± 3.2% and 1.0 ± 1.8%, respectively). To certify that oocytes were not undergoing maturation before the inhibition treatment, an additional control group was fixed immediately after aspiration, and 100% of these oocytes were at the GV stage.

Experiment 2

In experiment 2, the three different CDKIs were compared for meiotic inhibition efficiency (Table 2). Meiosis block of oocytes at GV stage was high for BOH and ROS (85.4 ± 1.9% and 79.9 ± 2.1%, respectively; $p > 0.05$), but was the highest for BL-I (97.5 ± 3.0%, $p < 0.05$). All treatments were superior to the control group (18.7 ± 7.7% of oocytes at the GV stage, $p < 0.05$). Correspondingly, few oocytes escaped meiotic inhibition and reached MII phase in treated oocytes (14.6 ± 1.9% and 17.5 ± 3.9% for BOH and ROS, respectively) while in the control group 78.5 ± 7.2% ($p < 0.05$) of the oocytes were in MII. For BL-I, none of the oocytes reached MII when compared with the other inhibitors ($p < 0.05$). Few oocytes were found in the intermediate stages of meiosis in any of the groups studied (0–2.8%). These results indicate that bovine oocytes can be maintained in meiotic inhibition *in vitro* for 24 h using BOH and ROS with similar efficiency, but BL-I shows an even more efficient meiotic block.

Experiment 3

To compare the reversibility of the treatments, oocytes were *in vitro* matured for 18 h after the 24 h blocking culture period. Control oocytes were only *in vitro* matured for 18 or 24 h. Results are presented in Table 3.

Nuclear meiotic maturation rates as a percentage of oocytes reaching MII were similar and high in all inhibition treatments (> 90%, $p > 0.05$). MII rates for

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Table 4 Embryo development of bovine oocytes fertilized *in vitro* after being submitted to meiotic inhibition by different CDKIs for 24 h and IVM for 18 h

Treatment	Oocytes No.	Cleavage No. (%)	Blastocysts* No. (%)
Control – 18 h	130	105 (80.6 ± 3.0)[a]	56 (43.4 ± 4.3)[a]
Control – 24 h	135	104 (77.1 ± 2.0)[a]	47 (35.0 ± 4.8)[b]
BOH 100 μM	133	105 (78.7 ± 4.8)[a]	27 (20.2 ± 2.4)[c]
ROS 25 μM	136	106 (77.8 ± 2.5)[a]	33 (24.2 ± 2.2)[c]
BL-I 100 μM	137	108 (78.9 ± 3.7)[a]	44 (32.3 ±2.3)[b]

Data are presented as the mean ± SEM of six replicates. Control – 18 h, 18 h IVM; control – 24 h, 24 h IVM; BOH, bohemine; ROS, roscovitine; BL-I, butyrolactone-I.
[a–c] Different letters within the same column indicate significant difference between treatments ($p < 0.05$).
* Blastocysts (D8) from total oocytes.

the treated groups were either similar (96.0 ± 2.1% and 93.6 ± 3.0% for ROS and BL-I, respectively; $p > 0.05$) or superior (96.9 ± 3.1% for BOH, $p < 0.05$) to the C24 group (91.0 ± 2.5%). All groups were superior to the C18 control group, which had 82.5 ± 1.2% of the oocytes in MII. This group had significantly more oocytes in intermediate stages of maturation (16.4 ± 1.9%, $p > 0.05$) than the other groups (3.1–8.0%, $p > 0.05$). These results indicate that meiotic block for all inhibitors is fully reversible in terms of meiotic nuclear maturation.

Experiment 4

Developmental rates of oocytes treated or not with different CDKIs are presented in Table 4. Cleavage rates were similar in all treatments (approximately 78%, $p > 0.05$) and were similar to both controls as well (80.6 ± 3.0% and 77.1 ± 2.0% for C18 and C24, respectively; $p > 0.05$). None of the treatments had any noticeable adverse effects on cleavage rates and were demonstrated to be reversible as well, since after meiotic inhibition oocytes matured *in vitro* and were able to be fertilized and to cleave.

On the other hand, later events of development as observed by blastocyst development were affected by some of the inhibiting treatments. BOH and ROS resulted in lower ($p < 0.05$) rates of blastocyst development (20.2 ± 2.4% and 24.2 ± 2.2%, respectively) when compared with both controls (43.4 ± 4.3% and 35.0 ± 4.8% for C18 and C24, respectively). BL-I had a developmental rate (32.3 ± 2.3%) similar to C24 ($p > 0.05$). C18 was superior (43.4 ± 4.3%, $p < 0.05$) to all groups, including C24. These results show an inhibitory effect of BOH and ROS later in development, since blastocyst development was reduced though the cleavage rates were similar in all groups.

Discussion

BOH was able to induce meiotic inhibition (over 65% of oocytes at GV stage), and increasing the concentration of the drug from 50 to 100–150 μM also increased rates of meiotic inhibition (over 80%).

Similar behaviour was observed by Mermillod *et al*. (2000) for ROS, which showed increased efficiency (over 80%) with increasing drug concentration. Kubelka *et al*. (2000) made similar observations for BL-I (inhibition rates from about 65% using 50 μM to 89% using 100 μM). ROS and BOH are CDKIs from the same family and act by inhibiting the specific cell-cycle-dependent kinases cdc2, cdk2 and cdk5 (Veselý *et al*., 1994; Meijer *et al*., 1997; Alberio *et al*., 2000), while BL-I acts as a selective inhibitor of cdk2 and cdc2 kinases (Kitagawa *et al*., 1993) with little effect on other kinases. These inhibitors act by competing for the ATP binding pocket of the kinase (Damiens & Meijer, 2000). BL-I and ROS would prevent meiosis resumption by inhibiting MPF activation (Kubelka *et al*., 2000; Krischek & Meinecke, 2001). There are no data regarding the action of BOH on MPF during meiotic inhibition in oocytes. However, it has been observed that BOH associated with ionomycin for activation of bovine oocytes maintained MPF at low levels (Alberio *et al*., 2000). This observation, together with the results of this study (GV block) and the results with similar inhibitors (BL-I and ROS), suggests that BOH would be maintaining MPF inhibition in bovine oocytes.

Rates of meiotic inhibition using ROS and BL-I in this study (80–98%) were similar to the results obtained by Mermillod *et al*. (2000) for ROS (83%) and by Kubelka *et al*. (2000) and Hashimoto *et al*. (2002) for BL-I (90% and 87%, respectively). BOH showed similar results (85% of oocytes at GV stage), demonstrating that the inhibitors are effective in blocking meiosis in bovine oocytes. However, BL-I showed a better response in blocking meiosis than BOH and ROS. Le Beux *et al*. (2003) did not observe any differences between ROS and BL-I regarding meiotic block efficiency, but comparisons between studies is difficult since these authors used a different species (pig) and different concentrations of the drugs (50 μM ROS and BL-I, while 25 μM ROS and 100 μM BL-I were used in this study).

Regarding reversal of meiotic inhibition, results from experiment 3 (94–97% of oocytes in MII) were similar to those obtained by Mermillod *et al*. (2000) for ROS (89%) and Lonergan *et al*. (2000) for BL-I (93.6%). IVM for reversal of meiotic inhibition lasted a period of 24 h in these reports, while in our experiments the IVM period was shorter (18 h) but equally effective. In our 18 h maturation control group, significantly fewer oocytes had reached metaphase II (82%), and about

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16% of the oocytes were still at intermediate stages of meiosis. Other authors have observed up to 80% of oocytes reaching MII after 18–20 h IVM (Costa *et al*., 1997; Lonergan *et al*., 1997; Khatir *et al*., 1998), similar to the percentage observed in this experiment. On the other hand, oocytes submitted to meiotic inhibition and then *in vitro* matured for only 18 h had MII rates similar or even superior (94–97%) to the control group matured for the usual 24 h (91%). Although no maturation kinetics studies were carried out, this observation would indicate that meiotic maturation is accelerated after a period of inhibition.

Ponderato *et al*. (2001), using a combination of ROS and BL-I at low concentrations, obtained 80.2% of oocytes in MII after 16 h IVM. Lagutina *et al*. (2002) observed that 82.5% of oocytes treated with roscovitine reached maturation after 16 h IVM. The same observation was made by Hashimoto *et al*. (2002) using butyrolactone I (> 85 % oocytes in MII at 18 h IVM). In our work more than 90% of meiosis-inhibited oocytes were in MII after 18 h IVM, in agreement with previous observations by others.

Embryo development rates after meiotic inhibition were 20%, 24% and 32% for BOH, ROS and BL-I respectively, and similar to those previously reported for ROS (31%, Mermillod *et al*., 2000; 25%, Lagutina *et al*., 2002) and BL-I (27%, Lonergan *et al*., 2000). However, in the studies of Mermillod *et al*. (2000) and Lagutina *et al*. (2002), rates for embryos exposed to ROS did not differ from those of control oocytes as seen in our study. This difference between laboratories could be a result of variations in culture conditions. There are no data on BOH, but it seems to have detrimental effects on development, possibly due to differences in its biochemical properties which are only observed later on in development.

The better performance of BL-I could be attributed to its better and more uniform meiotic inhibition (98%) than ROS (80%) and BOH (85%), which might have later implications during embryo development. Considering that in ROS and BOH treatments there would be a proportion of oocytes escaping meiotic inhibition (18% and 15% of oocytes in MII, respectively, by the end of inhibition culture) and, therefore, initiating meiotic progression, it would be expected that some of these oocytes would have been in nuclear maturation for a very long period of time, encompassing the inhibition and the maturation culture period (up to 42 h). A proportion of these oocytes would have been aged *in vitro*, prior to IVF and IVC. *In vitro* aging of oocytes is known to decrease subsequent embryo development, mainly by disrupting elements of the cytoskeleton (Kim *et al*., 1996). BL-I treatment, on the other hand, resulted in very few oocytes escaping the block (2.6%) and suffering the aging process, resulting in better developmental results.

The difference between inhibitors regarding their effects on the oocytes could be due to biochemical differences. Although they show common properties, they also exhibit chemical diversity (Knockaert *et al*., 2002). For example, olomoucine and roscovitine, which are very closely related compounds, present slight differences in the orientation of their purine ring with respect to the protein (Meijer & Kim, 1997) and roscovitine is more selective and more potent than olomoucine (Meijer *et al*., 1997). Different cellular responses have also been observed for bohemine and olomoucine, with bohemine being more effective than olomoucine at inhibiting growth and proliferation of prostatic cell cancer lines (Mad'arová *et al*., 2002). Roscovitine was also shown to be more efficient than olomoucine at inhibiting maturation in *Xenopus* oocytes (Flament *et al*., 2000).

Although BL-I although showed good results, similar to those of control oocytes matured for 24 h, there was a decrease in development in comparison with control oocytes matured for 18 h. It is possible that the concentrations used and/or the medium are still not adequate for inducing a reversible nuclear maturation block without compromising cytoplasmic maturation. Ponderato *et al*. (2001) have shown that by combining the two inhibitors BL-I and ROS it is possible to induce a reversible block with very low concentrations of these drugs, resulting not only in good blastocyst development but also in normal fetal development during early organogenesis (Ponderato *et al*., 2002). Wrenzycki *et al*. (2003) have shown that this same combination has less pronounced effects on mRNA expression of treated oocytes. Better adjustments in concentration of inhibitors, drug combinations and/or culture conditions might be necessary to improve the efficiency of these drugs. Recently, Hashimoto *et al*. (2002) have obtained a significant increase in blastocyst development (47.5% vs 33.5% in controls) and blastocyst cell numbers (147 vs 102 cells in controls) using 100 μM BL-I, after manipulating culture conditions by adding FCS instead of BSA and decreasing oxygen tension to 5% during inhibition culture.

One interesting observation was that the in the control groups, C18 oocytes showed better blastocyst development than C24 oocytes (43.1% vs 34.8% for C18 and C24 respectively), indicating that a shorter culture period might be sufficient for oocyte maturation. If maturation is achieved by most oocytes by 18 h (over 80%), it is possible that the majority would be somewhat aged by 24 h in culture (6 more hours after reaching maturation plus the hours until fertilization), compromising the cytoskeleton and, therefore, decreasing subsequent development (Kim *et al*., 1996).

In conclusion, in this study we have demonstrated that (a) BOH can effectively block meiosis in a reversible

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fashion, (b) BL-I is the most effective CDKI at inducing reversible meiotic and (c) embryo development to the blastocyst stage after meiotic inhibition is either maintained (BL-I) or decreased (BOH and ROS) by CDKIs.

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