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The effect of temporary meiotic attenuation on the in vitro maturation outcome of bovine oocytes

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Abstract The aim of this study was to investigate the effect of delaying maturation by extended culture of immature bovine oocytes in prematuration medium (PMC) containing single maturation inhibitor on their meiotic resumption and embryonic development. Bovine immature oocytes were cultured in M199 containing 10 μM of either inhibitor (roscovitine, cilostamide, or forskolin) for either 72 or 120 h followed by up to 48 h in maturation media supplemented with 7.5 IU follicle-stimulating hormone (FSH)/luteinizing hormone (LH). Two control groups were used. In untreated control, immature oocytes were cultured in the same medium as the experimental group without any inhibitors. In the FSH/LH control group, oocytes were cultured directly in the maturation medium supplemented with FSH/LH up to 48 h. In vitro matured oocytes were then inseminated with frozen–thawed bull sperm. Fertilization, defined as two-cell division 48 h post-insemination, and blastocyst formation were recorded. Total maturation rate for the 72-h group was 73%, 70%, 66%, and 69% for roscovitine, forskolin, cilostamide, and FSH/LH control, respectively, with no significant difference indicating that inhibitors have no negative effect on the oocyte maturation rate. The total fertilization rate for the 72-h group revealed that cilostamide (47%) and roscovitine (35%) were significantly higher than FSH/LH control (20%). The total blastocyst formation rates per inseminated oocytes revealed that among treatment groups, roscovitine (20%) had significantly higher rate than forskolin (9%). Overall, 72-h exposure period had better outcomes than 120 h in all the treated groups. In conclusion, prematuration culture of the bovine oocytes in the presence of maturation inhibitor for 72-h period at 10 μM concentration is sufficient in improving the bovine oocyte developmental competence.

Keywords Oocyte maturation · Meiotic inhibitors · Bovine oocyte · Infertility · IVM

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Introduction

In vitro maturation of oocytes (IVM) has been offered as an alternative to the traditional controlled ovarian stimulation and in vitro fertilization. However, in vivo matured oocytes are more competent than in vitro matured ones in producing better quality embryos and hence higher pregnancy outcome (Trounson *et al.* 2001). Competent oocytes have synchronized nuclear and cytoplasmic maturation, a necessary process for successful fertilization and subsequent developments (Edwards 1965a).

Cytoplasmic maturation, a poorly understood physiological process, supports the fertilization of the oocyte and early embryonic development. This process comprises accumulation of mRNA and proteins, redistribution of the organelles, and cellular metabolic changes during preovulatory follicle stage (Edwards 1965b). The morphological nuclear maturation alone does not necessarily indicate cytoplasmic maturation, hence developmental competence.

In vitro maturation of oocytes retrieved from the antral follicles has a shorter period of growth than preovulatory follicles in the conventional in vitro fertilization (IVF)

(Miller *et al.* 2000; Teissier *et al.* 2000; Cekleniak *et al.* 2001). During the preovulatory period, oocyte meiosis resumption is dependent on the level of cyclic adenosine monophosphate (AMP), produced by somatic granulosa cells and passed through the gap junctions to inhibit premature resumption (Mehlmann 2005). Cyclic AMP activates the cAMP-dependent protein kinase (PKA) by releasing two active catalytic subunits (Rose-Hellekant and Bavister 1996).

Maintaining high level of oocyte intracellular cAMP is facilitated by two mechanisms of production of cAMP, and prevention of its degradation. cAMP analogues (dbcAMP) (Kim *et al.* 2008), adenlylate cyclase activators (forskolin) (Bilodeau *et al.* 1993; Shu *et al.* 2008), and invasive adenyl cyclase (Aktas *et al.* 1995a, b) was used in production of intracellular cAMP during in vitro maturation of oocyte to maintain intracellular cAMP. These studies were able to show that high level of cAMP and PKA is responsible for preventing meiotic resumption. Phosphodiesterase enzyme inhibitor family including (cilostamide, milirnone, and Org9935) has shown to effectively maintain high level of intracellular cAMP by preventing its degradation by phosphodiesterase enzyme, hence attenuating meiotic resumption in porcine (Laforest *et al.* 2005), mouse (Tsafriri *et al.* 1996; Nogueira *et al.* 2003a), macaque (Jensen *et al.* 2002), bovine (Mayes and Sirard 2002; Albuz *et al.* 2010), and human oocytes (Nogueira *et al.* 2003b).

Ovulation by rupturing of the leading follicle in response to gonadotropin surge is associated with nuclear maturation by resumption of meiosis which is characterized by germinal vesicle breakdown (GVBD) and extrusion of first polar body. The nuclear maturation process is controlled by activity of the maturation-promoting factor (MPF). MPF plays an important role in GVBD, chromatin condensation, formation, and organization of microtubules (Brunet and Maro 2005). Multiple phosphorylation cascades and second messenger signaling pathways triggered by gonadotropin surge are very important for oocyte maturation. These phosphorylation cascades are activated by MPF and mitogen-activated protein kinase (MAPK) which is believed to be involved in chromatin condensation and microtubule formation (Fan and Sun 2004).

MPF levels fluctuate throughout the maturation process: it reaches to the highest levels at the time of GVBD and the end of first meiotic division to maintain metaphase II stage till sperm penetration (Sugiura *et al.* 2006). Roscovitine is a meiotic inhibitor that blocks MPF activation and can maintain the GVBD for 24–66 h (Oussaid *et al.* 2000; Coy *et al.* 2005a). It has been shown that roscovitine has no harmful effect on the embryonic and fetal development of animals (Coy *et al.* 2005a, b; Choi *et al.* 2006).

Accelerated nuclear maturation is a major problem in the IVM field when the oocytes are subjected to the in vitro environment, resulting in 24–48-h maturation following in vitro culture which may be responsible for poor outcome for downstream fertility treatments. Therefore, the aim of this study is to evaluate the effect of slowing the maturation process to better mimic the in vivo milieu utilizing maturation inhibitors including cilostamide, forskolin and roscovitine (maturation) inhibitors for 72 or 120 h prior to culturing in maturation medium to investigate the effect of extended prematuration culture on the maturation rate and subsequent development.

Materials and Methods

Oocyte source. Immature bovine oocytes were purchased from Applied Reproductive Technology Company (Madison, WI). They were aspirated from early antral follicles (2–6 mm) and selected based on compactness of cumulus. A total of 1,133 were shipped to our institution in pre-labeled tubes containing the meiotic inhibitors incubated at 37°C. Incubation during shipment was included total PMC period.

Twenty-five to 30 oocytes were used in each group, and the experiment was replicated four times to achieve the targeted sample size for each group.

Chemicals and reagents. All inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). M199 medium (Invitrogen, Carlsbad, CA) supplemented with 10% serum protein substitute (Origio, Trumbull, CT) and 100 U/ml penicillin and 100 μg/ml streptomycin (Pen Strep, Invitrogen) was used as base medium. Prematuration culture medium (PCM) consisted of base medium containing a final concentration of 10 μM of one of meiotic inhibitors of phosophodiesterase 3A (cilostamide), adenylate cyclase activator (forskolin), and MPF inhibitor (roscovitine). The complete maturation medium (CMM) was base medium containing 7.5 IU follicle-stimulating hormone (FSH)/luteinizing hormone (LH) (Menopure) (Ferring, San Diego, CA).

Experimental design. The immature oocytes were cultured in PMC containing either of each inhibitor (total of three PMC cultures) for either 72 or 120 h before culturing in CMM in a 37°C and 5.5% CO_2 in humidified air for 48 h. There were two controls. In untreated control (inhibitor-free) group, the immature oocytes were cultured in base medium for same PMC duration, while in FSH/LH control group, they were cultured in CMM medium for up to 48 h. Following CMM culture, the oocytes were checked for maturity, and those matured oocytes were inseminated with frozen–thawed bull sperm overnight (COBA Inc., Columbus, OH). Maturation was defined by cumulus expansion and extrusion of first polar body. The inseminated oocytes were washed from sperm and denuded mechanically to remove cumulus cell and culture in base medium for 24 h. They were then checked for fertilization, defined as two-cell embryos. It is very difficult to

visualize pronuclear formation (as a sign of fertilization) in unstained bovine oocyte as cytoplasm is dark brown. The two-cell embryos were then cultured for 8 d, and their development into blastocysts was monitored. Cleaved embryos were transferred to a fresh cleavage media every 2 d (Fig. 1). The whole culture period was under 37°C and 5.5% CO_2 in humidified air incubators.

Statistical analysis. Chi-square and Fischer's exact test statistical analysis were used to evaluate differences between groups using Vassarstats website for statistical analysis (http://faculty.vassar.edu/lowry/VassarStats.html). The significance level of the test was targeted at 0.05 (P value is <0.05). The sample size was calculated using the two-sided Z test with pooled variance.

Results

A total of 1,133 cumulus-enclosed bovine oocytes were used in this study to evaluate the effect of 72- and 120-h exposure to either maturation inhibitors of roscovitine, cilostamide, or forskolin on the in vitro maturation outcome of the immature oocytes.

Maturation rates of the oocytes after exposure to the meiotic inhibitors. Spontaneous maturation (matured while oocyte exposed to inhibitors), maturation (at 24 and 48 h) in complete maturation medium, total maturation (combination of maturation at 24 and 48 h), and overall maturation (spontaneous maturation + total maturation) were monitored in this study.

Spontaneous maturation. The efficacy of the meiotic inhibitors was evaluated by monitoring spontaneous maturation rate of immature oocyte following exposure to meiotic inhibitor prior to culturing in maturation media (Table 1). There was no significant difference in spontaneous maturation rate among inhibitors groups at 72-h exposure; however, when compared to non-treated control, all inhibitors groups had a significant lower rate of spontaneous maturation indicating their effectiveness in maintaining meiotic arrest of immature oocyte (P<0.05).

Spontaneous maturation for 120-h exposure groups showed no significant difference between treatment groups. However, untreated control had significantly higher rates indicating the inhibitors' effectiveness to attenuate the maturation process (P<0.05; Table 2).

Comparing each inhibitor at two different time exposures of 72 and 120 h (Tables 1 and 2) revealed no significant difference for cilostamide and forskolin (P>0.05). However, exposure to roscovitine for 72 h had significantly higher efficacy than 120 h (P=0.006).

Maturation. Following prematuration culture (72 or 120 h), immature oocytes were cultured in CMM medium for up to 48 h. In the 72-h PMC period, roscovitine group had a significantly higher maturation rate than all treatments and control groups at 24-h culture (Fig. 2; P<0.05).

Cilostamide, forskolin, and FSH/LH control had significantly higher maturation rate than the untreated control at 48-h culture (P<0.05). However, these rates are not significant when compared to roscovitone (P>0.05; Table 1). In the 120-h PMC period, oocyte maturation rate in the first 24 h of CMM culture, roscovitine- and cilostamide-treated groups were significantly lower than FSH/LH-treated control (P<0.05). However, it was not significantly different with forskolin (P>0.05; Fig. 3).

The maturation rate at 48 h CMM after 120 h PMC for cilostamide group was significantly higher than both FSH/

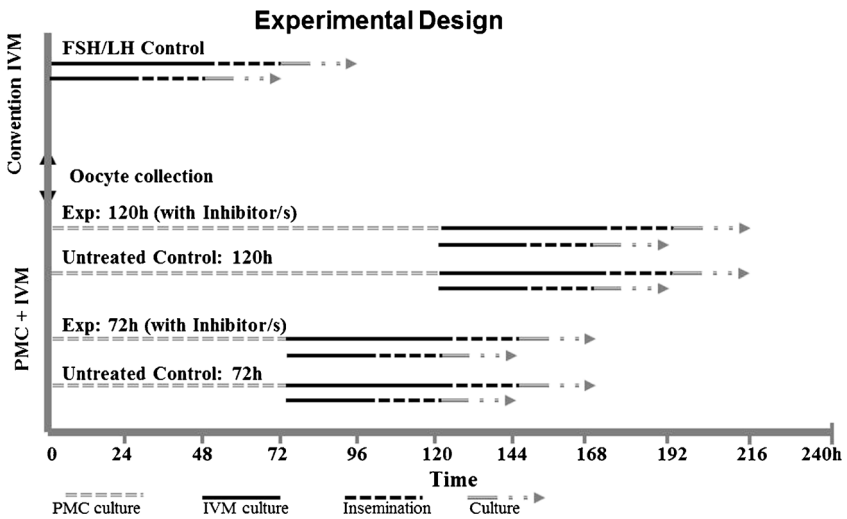

Figure 1 Diagram of experimental design.

Table 1 Maturation rates of oocyte cultured for 72 h in prematuration medium

72 h	Roscovitine	Cilostamide	Forskolin	Untreated control	FSH/LH control
N	108	117	105	113	125
Spontaneous maturation	19 (18%)	18 (15%)	23 (22%)	52 (46%)	N/A
No. matured (%)	79 (73%)**	78 (66%)**	74 (70%)**	45 (39%)	86 (69%)**
24 h (%)	60 (55%)*	47 (40%)*	46 (43%)*	39 (34%)*	50 (40%)*
48 h (%)	19 (17%)	31 (26%)	28 (26%)**	6 (5%)	36 (28%)**
Overall maturation	98 (91%)*	96 (82%)*	97 (92%)*	97 (85%)*	86 (69%)*
Wastage oocytes	10 (9%)	21 (18%)	8 (7%)	16 (14%)	29 (31%)

*$P<0.05$, **$P>0.05$

LH and untreated controls ($P<0.05$). However, roscovitine and forskolin groups were not significantly different from the FSH/LH group.

The maturation rate of the cilostamide group was significantly higher than forskolin ($P<0.05$). However, there was no significant difference between roscovitine and cilostamide or forskolin ($P>0.05$).

Roscovitine had a significantly higher maturation rate in the 24 h of the 72-h period than the 120 h ($P=0.001$). There was no significant difference in maturation rate following 24-h CMM culture for cilostamide and forskolin 72- and 120-h groups ($P>0.05$; Table 3).

Roscovitine- and cilostamide-treated group for 120 h had significantly higher maturation rate at 48 h than 72-h period (17 and 26%; $P<0.05$). On the other hand, forskolin group had lower rate for 120 h than 72-h group with no significant difference ($P>0.05$).

Total maturation. Total maturation rate was defined as combined maturation at 24- and 48-h CMM culture. There was no significant difference in maturation rate for the 72-h experimental and control groups indicating that inhibitors have no negative effect on the oocyte maturation rate ($P>0.05$; Table 1).

In 120-h exposure group, cilostamide had a significant higher rate of maturation than forskolin ($P<0.05$); however, this rate was not significant when compared with roscovitine ($P>0.05$). FSH/LH control has highest maturation rate among all groups, although it is not significant if compared with cilostamide ($P>0.05$), indicating that longer prematuration culture (120 h) may have adverse effect on the meiotic resumption rather than 72 h (Table 2).

Evaluation of the effect of each inhibitor on total maturation at the different exposure time revealed that roscovitine and forskolin had significantly higher rate at 72 h than 120 h ($P<0.05$; Fig. 4). However, there was no significant difference between 72- and 120-h time exposure duration for cilostamide ($P>0.05$; Table 3).

Overall maturation. Overall maturation rate, defined as summation of spontaneous and total maturation, in 72-h time exposure was significantly lower for FSH/LH control in comparison with cilostamide, roscovitine, forskolin, and untreated control groups ($P<.005$; Table 1). We did not attempt to evaluate the competency of the spontaneously matured oocytes.

In 120-h exposure time, the overall maturation rate of the FSH/LH-treated control was significantly lower than the all treated control groups ($P<0.05$). However, the overall maturation of the roscovitine and cilostamide groups was significantly higher than the untreated control ($P<0.05$; Table 2).

There was no difference in the overall maturation of each treatment and control groups between 72- and 120-h exposure duration ($P<0.05$).

Table 2 Maturation rates of oocyte cultured for 120 h in prematuration medium

120 h	Roscovitine	Cilostamide	Forskolin	Untreated control	FSH/LH control
N	113	116	107	114	115
Spontaneous maturation (%)	38 (33%)	22 (19%)	31 (29%)	52 (46%)	N/A
No. matured (%)	68 (60%)**	82 (70%)*, **	61 (57%)*	36 (31%)	83 (72%)**
24 h (%)	38 (33%)*	42 (36%)*	40 (37%)**	25 (22%)	56 (48%)*, **
48 h (%)	30 (27%)	40c (34%)*	21 (19%)*	11 (9%)	27 (23%)*
Overall maturation	106 (93%)*	104 (89%)*	92 (86%)*	88 (77%)	83 (72%)*
Wastage oocytes	7 (6%)	12 (11%)	15 (14%)	26 (22%)	22 (18%)

*$P<0.05$, **$P>0.05$

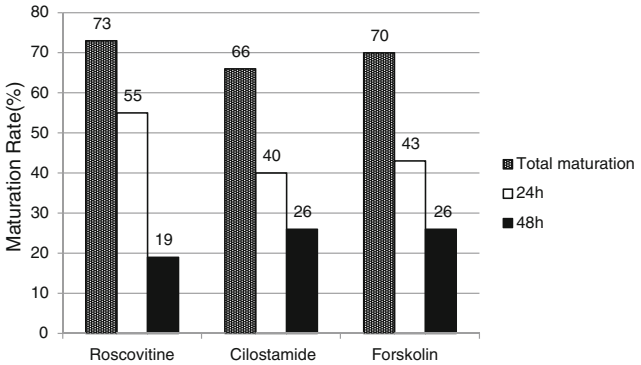

Figure 2 Total maturation rate and maturation rate (at 24 and 48 h) of oocyte cultured for 72 h in prematuration culture containing either inhibitor. Total maturation rate was 73%, 66%, and 70% for roscovitine, cilostamide, and forskolin with no significant difference ($P>0.05$). Roscovitine (55%) had significantly higher maturation rate at 24 h than cilostamide (40%) and forskolin (43%) ($P<0.05$). There was no significant difference in maturation rate at 48 h between roscovitone (17%), cilostamide (26%), and forskolin (26%) ($P>0.05$).

Oocyte wastage. Some of the oocytes did not resume meiosis, arrested, cleaved, or degenerated; they were considered wastage (original number of oocyte minus overall matured). Our data shows that FSH/LH control had significantly high oocyte wastage that other treatment and untreated group in 72-h exposure time. Among treatment groups, cilostamide had higher oocyte wastage than roscovitine and forskolin with no significant difference ($P>0.05$; Table 1).

In 120-h time exposure, there was no significant difference in oocyte wastage among experimental groups ($P>0.05$). However, roscovitine had significantly lower oocyte wastage rate than FSH/LH-treated and untreated controls ($P<0.05$). Comparison of each treatment group between two exposure times (72 and 120 h) reveled no significant difference in oocyte wastage rate.

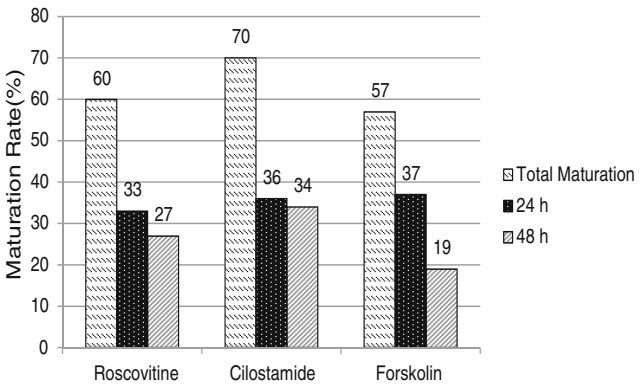

Figure 3 Total maturation rate and maturation rate (at 24 and 48 h) of oocyte cultured for 120 h in prematuration culture in the presence of either inhibitors. Cilostamide (70%) had a significant higher total maturation rate than forskolin (57%; $P<0.05$); however, this rate was not significant when compared with roscovitine (60%; $P>0.05$). There was not a significant difference in maturation rate at 24 h for roscovitine (33%), cilostamide (36%), and forskolin (37%; $P>0.05$). Cilostamide (37%) had significantly higher maturation rate than Foraskolin (19%) at 48 ($P<0.05$). However, it was not significant with Roscovitin (27%) ($P>0.05$).

Table 3 Comparison of maturations rates for each inhibitor at 72 and 120 h

Groups	72-h maturation rate	120-h maturation rate	*P* value
Roscovitine			
24 h	55%	33%	0.01
48 h	18%	27%	<0.0001
Total	73%	60%	0.02
Cilostamide			
24 h	40%	36%	0.1
48 h	26%	34%	0.2
Total	66%	70%	0.3
Forskolin			
24 h	46%	37%	0.4
48 h	28%	19%	0.4
Total	70%	57%	0.02

Fertilization rates. Matured oocytes were inseminated following 24- or 48-h culture in CMM according to their maturity. In 72-h exposure time, cilostamide had significantly higher fertilization rate than roscovitine, and FSH/LH control groups among the oocytes matured at 24 h ($P>0.05$; Table 3). However, this rate was not significant in comparison with forskolin. For those oocytes matured at 48 h, cilostamide and roscovitine had significantly higher fertilization rate than FSH/LH. However, it was not significant with forskolin (Table 4).

Evaluating total fertilization rate (total number of fertilized egg per total number of inseminated at 24 and 48 h) revealed that cilostamide and roscovitine were significantly higher than FSH/LH control ($P<0.05$). Among treatment groups, cilostamide was significantly higher than forskolin; however, it was not significant in comparison with roscovitine.

In 120-h exposure time, roscovitine had significantly higher fertilization rate than treatment groups of cilostamide and forskolin and FSH/LH control ($P<0.05$; Table 4) among oocytes matured at 24 h CMM culture. This rate was not significant between all treatment and FSH/LH control groups ($P>0.05$) for oocytes matured at 48 h.

Total fertilization rate for roscovitine was significantly higher than treatment and FSH/LH control groups ($P<0.05$; Table 5).

Comparing each treatment group between two time exposures of 72 and 120 h revealed that roscovitine had significantly higher fertilization rate in 120-h exposure time than 72 h for those matured at 24 h ($P<0.05$; Table 5). This rate was not significantly different for oocytes matured at 48 h between 72- and 120-h time exposure ($P>0.05$). There was no significant difference in total fertilization rate between

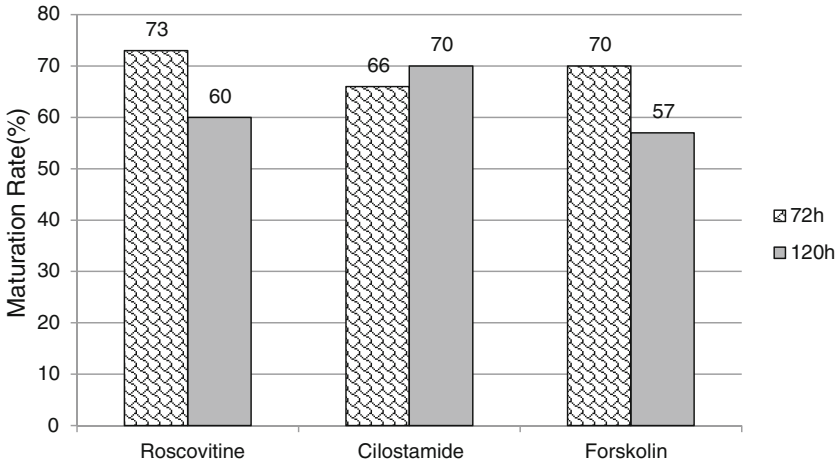

Figure 4 Comparison of total maturation at 72 and 120 h for each inhibitor. Roscovitine (73%) and forskolin (70%) had significantly higher total maturation rate at 72 h than 120 h (60 and 57% respectively) ($P<0.05$). There was no significant difference in this rate for cilostamide between 72-h (66%) and 120-h (70%) time exposure period ($P>0.05$).

these two time exposures ($P>0.05$). Cilostamide, on the other hand, had significantly higher fertilization rate at 24 h, 48 h, and total fertilization in 72 h than 120-h exposure time ($P<0.05$; Table 5). There were no significant differences in fertilization rates at 24 h, 48 h, and total fertilization for 72-h exposure time when compared to 120-h exposure time in forskolin treatment group ($P>0.05$; Table 6).

Blastocyst formation. The blastocyst formation rate was calculated based on fertilized and inseminated oocyte according to their maturation timing (24 and 48 h). Total blastocyst formation rate referred to the combination of blastocyst formation at 24 and 48 h.

In 72-h exposure time, blastocyst formation (24 and 48 h) rate per fertilized oocytes was not significantly different among roscovitine, cilostamide, forskolin, and FSH/LH control ($P>0.05$; Table 7). The total blastocyst rate per fertilized oocytes was not significantly different between all treatments and control groups ($P>0.05$).

The blastocyst formation rate per inseminated oocytes at 24 and 48 h was not significant among treatments and control groups ($P>0.05$; Table 7). Analyzing the total blastocyst formation rates per inseminated oocytes revealed that among treatment groups, roscovitine had significantly higher rate than forskolin ($P<0.05$). However, there was no significant difference between cilostamide and forskolin groups ($P>0.05$). Both roscovitine and cilostamide were significantly higher when compared with FSH/LH ($P<0.05$).

In 120-h exposure period, the blastocyst formation rate per fertilized oocytes at 24 h was not significantly different among roscovitine, cilostamide, forskolin, and FSH/LH control ($P<0.05$; Table 8). This rate was not significantly different between roscovitine, cilostamide, and FSH/LH control groups for those oocytes inseminated at 48 h. There was no embryo developed to blastocyst in forskolin treatment group at 48 h. Total blastocyst rate per fertilized oocytes was not significantly different between all treatments and control groups ($P>0.05$).

Blastocyst formation rate per inseminated oocytes at 24 h was not significantly different among all treatments and control groups ($P<0.05$; Table 9). This rate was not significantly different between treatments and control groups for those inseminated at 48 h. Total blastocyst rate per inseminated oocytes was significantly different between roscovitine and forskolin ($P<0.05$); however, this rate was not significant in comparison with cilostamide and FSH/LH controls ($P>0.05$).

Table 4 Fertilization rates in vitro matured oocyte cultured for 72 h in prematuration medium

72 h	Roscovitine	Cilostamide	Forskolin	FSH/LH control
No. inseminated				
Total	79	78	74	86
24 h	60	47	46	50
48 h	19	31	28	36
No. fertilized per no. inseminated				
Total (%)	28/79 (35%)***	37/78 (47%)*, ***	22/74 (29%)*	17/86 (20%)*
No. fertilized per maturation time				
24 h (%)	20/60 (33%)*	24/47** (51%)*, ***	16/46 (35%)***	12/50 (24%)*
48 h (%)	8/19 (42%)***	13/31 (41%)***	6/28 (21%)***	5/36 (14%)

*$P<0.05$, **$P<0.05$, ***$P>0.05$

Table 5 Fertilization rates in vitro matured oocyte cultured for 120 h in prematuration medium

120 h	Roscovitine	Cilostamide	Forskolin	FSH/LH control
No. inseminated				
Total	68	82	61	83
24 h	38	42	40	56
48 h	30	40	21	27
No. fertilized per no. inseminated				
Total (%)	29/68 (45%)*	20/82 (24%)*	13/61 (21%)*	24/83 (32%)*
No. fertilized per maturation time				
24 h (%)	22/38 (58%)*	14/42 (33%)*	11/40 (28%)*	16/56 (29%)*
48 h (%)	7/30 (23%)**	6/40 (15%)**	2/21 (9%)**	8/27 (30%)**

*$P<0.05$, **$P>0.05$

Comparing the blastocyst formation rates (per fertilized and per inseminated oocyte) for each inhibitor between two exposure times of 72 and 120 h revealed no significant difference for 24 h, 48 h, and total rate ($P>0.05$; Tables 9 and 10).

Discussion

The final steps of in vivo oocyte development are still a mystery; understanding of these steps might be the key element in developing an optimum IVM culture system. In human, the implantation failure and miscarriage rates in IVM cycles are higher than conventional IVF cycles (Nogueira *et al.* 2003b, 2006; Jurema and Nogueira 2006). It is postulated that poor early embryonic development is related to incorrect microtubule and chromatin condensation in addition to abnormal activation of the embryonic genome (Combelles *et al.* 2002; Schramm *et al.* 2003).

Table 6 The fertilization rate for each inhibitor in the 72-h and 120 PMC exposure periods

Groups	72-h fertilization rate	120-h fertilization rate	*P* value
Roscovitine			
24 h	33%	58%	0.02
48 h	42%	23%	0.07
Total	35%	45%	0.2
Cilostamide			
24 h	51%	33%	0.01
48 h	41%	15%	0.02
Total	47%	24%	0.01
Forskolin			
24 h	35%	28%	0.1
48 h	21%	9%	0.3
Total	29%	21%	0.2

In vitro matured oocytes presumably have an incomplete maturation due to deficiency of full cytoplasmic maturation (Mayes *et al.* 2007) which could be the explanation of the clinical unsatisfactory outcome. Consequently, extending meiotic arrest period by utilizing meiotic inhibitors may be beneficial for nuclear and cytoplasmic synchrony (Lonergan *et al.* 1997; Avery *et al.* 1998; Nogueira *et al.* 2003a).

In the present study, three types of meiotic inhibitors were used in PMC (phase I) for 72 or 120 h followed by up to 48 h of CMM (phase II) culture. The forskolin inhibitor is adenylate cycle enzyme activator which is responsible for synthesis of the intracellular cAMP to maintain meiotic arrest by keeping the intracellular cAMP to the level that prevents the GVBD. In the bovine oocytes, the adenylate cyclase activity has been proven to be localized in cumulus cells and the plasma membrane, suggesting culturing intact cumulus oocyte complex (without denudation) may have better maturation outcome (Kuyt *et al.* 1988; Bilodeau *et al.* 1993; Nogueira *et al.* 2006).

Cilostamide, a phosphodiesterase (PDE) 3 A inhibitor (a specific PDE isoenzyme), inhibits the action of the PDE by functioning as active site competitor with lower side effects, preventing degradation of cAMP in the intracellular component. Cilostamide is effective in keeping the cumulus oocyte gap junctions intact, thus allowing more time for metabolic exchange between the oocytes and the media which is completely broken in the spontaneous maturation process (Thomas *et al.* 2004a, b; Albuz *et al.* 2010).

MPF is composed of the two catalytic subunits p43 and cdc2 in addition to a regulatory subunit cyclin B. The activation of this factor is by binding of the cyclin B to the cdc2 and phosphorylation of threonine 161 and tyrosine 15. Roscovitine is a cyclin-dependant kinase inhibitor that can suppress the activity of MPF by blocking the action of cyclin B. MPF has an effect on both early and late stages of maturation; addition of roscovitine to the culture media before GVBD and 24 h after GVBD showed meiotic controlling effect on the oocytes (Zhang *et al.* 2011).

Our data revealed that roscovitine (MPF inhibitor) has superiority over the other two inhibitors by having the highest maturation rates among, second best fertilization rate (after cilostamide), and highest blastocyst formation rates. Cilostamide (PDE-3A inhibitor) comes second in its maturation rate after roscovitine with better fertilization but lower blastocyst formation rates. However, forskolin comes in last among all inhibitors with poor maturation and developmental outcomes. It was observed that the FSH/LH control group had

Table 7 Blastocyst formation rates in vitro matured oocyte cultured for 72 h in prematuration medium

72 h	Roscovitine	Cilostamide	Forskolin	FSH/LH control
No. fertilized (%)	28/79 (35%)	37/78 (47%)	22/74 (29%)	17/86 (20%)
24 h (%)	20/79 (25%)	24/78 (31%)	16/74 (22%)	12/86 (14%)
48 h	8/79 (10%)	13/78 (16%)	6/74 (7%)	5/86 (6%)
No. blastocyst/fertilized				
24 h (%)	12/20a (60%)	11/24b (46%)	6/16 (38%)	5/12 (42%)
48 h (%)	4/8 (50%)	3/13 (23%)	1/6 (16%)	1/5 (20%)
No. blastocyst/total fertilized	16/28 (57%)*	14/37 (37%)*	7/22 (31%)*	6/17 (35%)*
No. blastocyst/no. inseminated				
24 h (%)	12/60 (15%)	11/78 (14%)	6/74 (8%)	5/86 (6%)
48 h (%)	4/19 (5%)	3/78 (4%)	1/74 (1%)	1/86 (1%)
No. blastocyst/total inseminated	16/79 (20%)*, **	14/78 (18%)*	7/74 (9%)**	6/86 (7%)**

*$P>0.05$, **$P<0.05$

a higher oocyte wastage rate in comparison to all the treated groups which leads us to believe that the current IVM system (which does not utilize inhibitors) is not the optimal on evident by the oocyte wastage.

Our results indicated that 72-h prematuration culture period is more optimum than 120-h exposure due to better outcome in terms of meiotic resumption, fertilization, and blastocyst formation rates at 10 μM concentration of inhibitors.

Keeping the cumulus intact during the whole period of (PMC) has proved to be very effective as it plays a key role in either transporting or processing the inhibitory factors produced by the theca cell monolayer (Richard and Sirard 1996; Colleoni *et al.* 2004). Maintaining the high level of intracellular cAMP by exposing immature oocyte to adenylate cyclase activator (forskolin) or PDE-3A inhibitor points to possible cAMP role in regulating the meiotic process in the bovine oocytes (Hashimoto *et al.* 2002; Thomas *et al.* 2004b). It seems that concentration of 10 μM is sufficient to maintain meiotic arrest as there was no significant difference between 10 and 50 μM reported by Mario and Marc-André (2002) In human, Nogueira et al. reported 67% for both maturation and fertilization rates with intra-cytoplasmic sperm injection (ICSI) after a 24-h period of (PMC) (Nogueira *et al.* 2006) supporting the idea of temporary meiotic attenuation for human application. More recently, Yi-min Shu et al. extended the (PMC) period to 48 h and compared the effect of cilostamide, forskolin, and their combination on the IVM outcome of human oocytes in a controlled ovarian hyperstimulation cycles, reporting favorable effect of the combined treatment in terms of maturation rate (65.4%) and fertilization (76.4%) using ICSI over any of them alone (Shu *et al.* 2008). Mermillod et al. reported that no negative effect on the developmental outcome of oocytes after culture for 24 h with 25 μM roscovitine (Mermillod *et al.* 2000). Others reported adverse outcomes after exposure to higher concentration (>50 μM) for 24 h (Donnay *et al.* 2004; Kaedei *et al.* 2010). Coy et al. reported that roscovitine with doses ranging from 12.5 to 50 μM was not harmful in terms of zona pellucida hardening, nuclear maturation, fertilization, and blastocyst formation after culture for 48 h. However, they concluded that this period is not sufficient to improve the in vitro

Table 8 Blastocyst formation rates in vitro matured oocyte cultured for 120 h in prematuration medium

120 h	Roscovitine	Cilostamide	Forskolin	FSH/LH control
No. fertilized (%)	29/68 (45%)	20/82 (24%)	13/61 (21%)	24/83 (32%)
24 h (%)	22/68 (32%)	14/82 (17%)	11/61 (18%)	16/83 (19%)
48 h (%)	7/68 (13%)	6/82 (7%)	2/61 (3%)	8/83 (9%)
No. blastocyst/fertilized				
24 h (%)	10/22 (45%)	8/14 (57%)	4/11 (36%)	4/16 (25%)
48 h (%)	2/7 (28%)	2/6 (33%)	0/2 (0%)	5/8 (63%)
No. blastocyst/total fertilized (%)	12/29 (41%)*	10/20 (50%)*	4/13 (31%)*	9/24 (38%)*
No. blastocyst/no. inseminated				
24 h (%)	10/68 (15%)	8/82 (9%)	4/61 (6%)	3/83 (3%)
48 h (%)	2/68 (3%)	2/82 (3%)	0/61 (0%)	5/83 (8%)
No. blastocyst/total inseminated	12/68 (18%)**	10/82 (12%)*	4/61 (6%)**	9/83 (11%)*

* $P>0.05$, ** $P<0.05$

Table 9 Comparison of blastocyst formation rates per fertilized oocytes in 72- and 120-h exposure groups

Groups	72-h blastulation rate	120-h blastulation rate	*P* value
Roscovitine			
24 h	60%	45%	0.2
48 h	50%	28%	0.3
Total	57%	41%	0.23
Cilostamide			
24 h	46%	57%	0.4
48 h	23%	33%	0.5
Total	37%	50%	0.6
Forskolin			
24 h	38%	36%	0.6
48 h	16%	0%	0
Total	31%	31%	1

maturation outcome (Coy *et al.* 2005a) supporting our hypothesis to extend the duration of (PMC). Albuz et al. reported excellent results by 1–2-h culture of immature bovine oocyte harvested from intermediate antral follicle in the presence of adenylate cyclase activator (forskolin) and IBMX (a nonspecific PDE inhibitor), followed by cilostamide and FSH. They cultured in serum-free condition with 69% blastocyst formation rates (Albuz *et al.* 2010). In our study, we used oocyte from early antral follicle and cultured them in the presence of inhibitors in the PMC phase only (not the IVM phase) with lower maturation rate than Albuz et al. Our low maturation rate could be related to the source of oocyte as they were in earlier maturation and require longer time to mature.

It was observed in our study that 80% of the blastocysts resulted from oocytes matured at 24 h of culture in maturation medium suggesting that the earlier the oocytes resume meiosis the better developmental outcome. Our data shows that 120-h prematuration culture at 10 μM concentration of inhibitors may have a negative impact on the oocyte maturation rate at 24 h of CMM culture and require longer time to resume meiosis in comparison to 72-h period. We did not attempt to lower concentration of inhibitors for longer periods; however, it may have positive impact on the matured oocytes.

Table 10 Comparison of blastocyst formation rates per inseminated oocytes in 72- and 120-h exposure groups

Groups	72-h blastulation rate	120-h blastulation rate	*P* value
Roscovitine			
24 h	15%	15%	1
48 h	5%	3%	0.9
Total	20%	18%	0.9
Cilostamide			
24 h	14%	9%	0.2
48 h	4%	3%	0.4
Total	18%	12%	0.3
Forskolin			
24 h	8%	4%	0.5
48 h	1%	0%	0
Total	9%	4%	0.4

Prematuration culture of the bovine oocytes with maturation-promoting factor or phosphodiesterase-3A inhibitor for 72-h period at 10 μM concentration is sufficient in improving the bovine oocyte developmental competence. If applicable to human, this may improve the overall in vitro maturation outcome.

Authors' contributions Tarek Farghaly, MD participated in study design, execution, analysis, manuscript drafting, and critical discussion. Prof. Essam-Aldein M. Khalifa, MD participated in the study design and manuscript drafting. Prof. Sayed M. Mostafa, MD participated in the study design and manuscript drafting. Mostafa Hussien, MD participated in the study design and manuscript drafting. Mohamed A. Bedaiwy, MD participated in the study design and manuscript drafting. Ali Ahmady, PhD, HCLD participated in study design, analysis, manuscript drafting, and critical discussion.

Conflict of interest None

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