Original Article

The Effects of cAMP-elevating Agents and Alpha Lipoic Acid on *In Vitro* Maturation of Mouse Germinal Vesicle Oocytes

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Abstract

Background: In spite of extensive efforts to improve *in vitro* oocyte maturation, the obtained results are not very efficient. This study was conducted to assess impacts of cAMP elevating agents and alpha lipoic acid (ALA) on *in vitro* oocyte maturation and fertilization.

Methods: Mouse germinal vesicle (GV) oocytes were categorized into cumulus denuded oocytes (DOs; n=896) and cumulus oocyte complexes (COCs; n=1077) groups. GV oocytes were matured *in vitro* with or without ALA; (I) without the meiotic inhibitors; (II) supplemented with cilostamide; (III) supplemented with forskolin and (IV) supplemented with Forskolin plus cilostamide. The obtained metaphase II (MII) oocytes were subjected to *in vitro* fertilization. Independent-samples t-testand ANOVA were used for data analysis. A p-value less than 0.05 was considered to be statistically significant.

Results: The COCs maturation, fertilization and two cell embryo rates were higher than those of DOs in the control group, while no significant difference was observed between relevant COCs and DOs when they were cultured with cilostamide meiotic inhibitors in two step manner. Combined treatment of cilostamide and forskolin significantly elevated the developmental rates in both COCs and DOs as compared to other groups. The developmental rates of COCs and DOs in the presence of ALA were similar to their respective groups without ALA.

Conclusion: cAMP elevating agents were more effective on DOs than COCs with regard to rates of maturation and fertilization. However, ALA did not affect the developmental rates of both COCs and DOs in *in vitro* maturation in one or two step manner.

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Introduction

Synchronization of nuclear and cytoplasmic maturation is necessary for oocyte developmental competence in order to support fertilization and early embryo development (5–7). Despite nuclear maturation in the process of *in vitro* oocyte maturation, cytoplasmic maturation is threatened and incomplete which results in oocytes with compromised developmental competence (8–10). Since cytoplasmic maturation is achieved gradually during folliculogenesis and duration of *in vitro* maturation (IVM) process would not be fulfilled in this time period, *in vitro* conditions do not support optimal oocyte cytoplasmic maturation (11, 12).

It has been well documented that simultaneous maturation of nucleus and cytoplasm is partially provided by means of *in vitro* cessation of oocyte meiosis, leading to improved oocyte development competence (13–15). In this resting interval, the oocyte will find the opportunity to continue transcription of mRNA, post-translational modifications of proteins, relocation and modification of organelles which are essential to sustain normal fertilization and further embryonic development (16).

It has been revealed that cyclic adenosine monophosphate (cAMP) has a decisive role in maintaining mammalian oocyte meiotic arrest (17). Increased levels of cAMP potentiates cAMP dependent protein kinases (PKA) activity result in oocyte meiosis arrest due to inhibition of maturation-promoting factor (MPF) and mitogen activating protein kinase (MAPK) (18, 19). Adenylyl cyclase (AC) and phosphodiesterases (PDEs) are enzymes which organize oocyte meiotic arrest by regulating the intra-oocyte level of cAMP through its synthesis and degradation (20).

In fact, oocyte meiotic progression could be temporarily inhibited or attenuated via increasing intra-oocyte cAMP levels by cAMP-elevating agents such as PDE subtype inhibitors and/or AC activator in order to improve *in vitro* oocyte developmental competence in several species (14, 20, 24–28).

The critical role of cumulus cells on oocyte nuclear maturation and cytoplasmic maturation has been shown (30–33). It has been established that, cumulus cells have an important role in oocyte meiotic transition from prophase to metaphase (28, 34). Cumulus cells are the main source of cGMP and its transmission via gap junctions results in inhibiting phosphodiesterase 3A (PDE3A) which in turn leads to an increase in cAMP level and meiotic arrest (34). Luteinizing hormone (LH) directly causes a decrease in cGMP and indirectly in cAMP through disrupting the gap junction between cumulus cells and oocyte, in the oocyte that allows meiotic resumption (34, 35).

Among the various factors which affect the oocyte developmental competence, generation of oxidative stress (OS) in *in vitro* media is important (36). Any disturbance in the ratio of pro-oxidant and anti-oxidant could result in OS leading to generation of reactive oxygen species (ROS) and free radicals in *in vitro* culture media which affect outcomes of IVM (37, 38). Adding an antioxidant to *in vitro* culture media to improve the cultivation conditions is a general consensus (36). In this continuum, alpha lipoic acid (DL-6,8-thioctic acid; ALA) as a coenzyme of mitochondrial multienzyme complexes and its reduced form, dihydrolipoic acid (DHLA), are well-known for their antioxidant properties. It has been shown that ALA improves developmental competence of cultured mouse pre-antral follicles through decreasing ROS production and increasing total anti-oxidant capacity (38). Both ALA and DHLA can act through scavenging of the ROS such as hydroxyl radicals, superoxide radicals, peroxyl radicals, hypochlorous acid and singlet oxygen; chelating of zinc, copper and iron; and intracellular recycling of other antioxidants (39, 40).

In sum , the objectives of the present study were; 1) to assess the effects of cilostamide, a specific PDE3 inhibitor, and/or forskolin, an AC activator, in the two step *in vitro* culture manner on the oocyte maturation and fertilization in the presence or absence of cumulus cells, and 2) to determine whether adding ALA to culture medium supplemented with forskolin and cilostamide can increase cultivation period and modulate production of ROS in two step culture manner to improve the oocyte maturation and fertilization.

Methods

Reagents: All reagents were obtained from Sigma-Aldrich (Hamburg, Germany), unless stated otherwise and all media were made with Mili-Q water.

Animals: All the mice used belonged to Naval Medical Research Institute (NMRI), housed and bred in accordance with the Guide for Care and Use of Laboratory Animals of Damghan University.

Experimental Design: We chose two main groups of cumulus oocyte complexes (COCs) and cumulus denuded oocytes (DOs) to evaluate the effects of meiotic inhibitors (cilostamide and forskolin) and ALA on the maturation and fertilization of mouse germinal vesicle (GV) oocytes. GV oocytes from each main group were randomly distributed among four different subgroups: (I) IVM without inhibitors (control); (II) IVM with 10 μM cilostamide, (III) IVM with 50 μM forskolin and

(IV) IVM with 50 *µM* forskolin and 10 *µM* cilostamide. Each group was also cultured in the presence or absence of ALA (DL-6,8-thioctic acid). Totally, 16 experimental groups were studied.

Meiotic inhibitors were used in two step IVM manner. Briefly, in step I, GV oocytes were transferred to microdrops of IVM medium supplemented with meiotic inhibitor (cilostamide and/or forskolin) and then incubated for 18 hours. In step II, meiotic inhibitors were removed and subsequently transferred to IVM medium and cultivated for an additional 18 *hr*. The control group was cultured in maturation medium only for 18 *hr* without using any meiotic inhibitors. Based on the experimental group, 18 or 36 *hr* after the onset of cultivation, the maturation status of the oocytes in each group was examined and classified as GV, germinal vesicle breakdown (GVBD) or metaphase II (MII) while MII oocytes were submitted to IVF.

Isolation of GV Oocytes: Germinal vesicle oocytes were obtained from 8–10 week old female mice (n=40) who were primed with an intraperitoneal injection of 7.5 *IU* pregnant mare's serum gonadotropin (PMSG; Folligon®, Intervet, Castle Hill, Australia) 48 *hr* prior to oocyte retrieval. Mice were killed by cervical dislocation and then their ovaries were collected in HEPES-buffered TCM_{199} medium (Gibco BRL, Paisley, UK) supplemented with 10% (*v/v*) fetal bovine serum (FBS; Gibco BRL, Paisley, UK), 0.23 *mM* sodium pyruvate, 100 *IU/ml* penicillin and 75 *µg/ml* streptomycin. The COCs were achieved by puncture of antral follicles with sterile 29 gauge needles. A total of 1973 COCs were harvested. 1077 COCs with uniform covering of 3–5 layers of cumulus cells and homogenous cytoplasm were selected for COCs groups and the remaining (897) were prepared for DOs groups by repeated pipetting and flushing through a small fine controlled bore pipette. After washing the oocytes in fresh HEPES–buffered TCM_{199} medium, they were used as described in experimental design section. Collections of oocytes were performed at minimum possible time prior to transfer to maturation medium. Also, in order to prevent spontaneous maturation, oocytes of each experimental group were collected in the presence of meiotic inhibitors which were used in the same group.

In vitro maturation of GV oocytes: The basal maturation medium was TCM_{199} supplemented with 0.22 *g/L* $NaHCO_3$, 100 *IU/ml* penicillin and 75 *µg/ml* streptomycin, 0.23 *mM* sodium pyruvate, 10% FBS, 10 *ng/ml* epidermal growth factor (EGF), 75 *mIU/ml* rhFSH and 10 *IU/ml* hCG. Also according to the experimental design, 100 *µM* of ALA (DL-6,8-thioctic acid), 10 *µM* of cilostamide, 50 *µM* of forskolin and a combination of 10 *µM* of cilostamide plus 50 *µM* of forskolin were added to the maturation medium.

Cilostamide, forskolin and ALA were dissolved in dimethylesulphoxide (DMSO) at 100 *mM* concentration as the stock solution and protected from light and kept at −20*°C*. Before using, they were immediately diluted to the appropriate concentration in maturation medium to reach the final concentrations of 10, 50 and 100 *µM* respectively. Final concentrations of DMSO in maturation medium were 0.001% for cilostamide groups, 0.005% for forskolin groups and 0.01% for ALA groups. It has been shown that, the concentration of DMSO in the medium up to 0.1%, does not have any adverse effect on oocyte maturation progression (15).

Groups of five oocytes were cultured in 20 *µl* drops of maturation medium under mineral oil at 37*°C*, 100% humidity in 5% CO_2 for 18 *hr* or 36 *hr* according to the categorization of groups described above in experimental design. At the end of the culture period, the number of degenerated oocytes, oocytes at GV, GVBD and MII stages were counted using an inverted microscope with Hoffman modulation contrast equipment (Nikon, Tokyo, Japan). The oocytes at MII stages were collected and used for *in vitro* fertilization. Each experiment was repeated at least three times.

In vitro fertilization and embryo culture: Sperm was obtained from the dissected cauda epididymis of the mature NMRI male mice. Cauda epididymis was placed into a 500 *µl* drop of T6 medium with 5 *mg/ml* BSA under mineral oil (41). The epididymal contents were squeezed out by the use of forceps. Capacitation of spermatozoa was attained by allowing the drops containing freshly released spermatozoa to stay at 37*°C* in 5% CO_2 and 95% humidity incubator for 90 *min*. Capacitated sperm suspension was added to 20 *µl* drops of fertilization medium which consisted of T6 medium supplemented with 15 *mg/ml* BSA to give the final motile sperm concentration of $1–2\times10^6$/*ml* and sperm number was calculated as described previously (42). Five MII oocytes were collected from different groups and separately transferred to each drop of IVF medium. MII oocytes and spermatozoae were incubated for 6 *hr* at 37*°C* in 5% CO_2 and 95% humidity incubator, and then they were removed from the fertilization medium, and rinsed 3 times with T6 medium+5 *mg/ml* BSA. Presumptive zygotes were further cultured in 10 *µl* drops of T6 medium with 5 *mg/ml* BSA and incubated at 37*°C* in humidified 5% CO_2 incubator. After the 4 *hr* incubation for fertilization and the following 3 *hr* of culture in fresh medium, zygotes were evaluated for pronucleus formation by using an inverted microscope. The number of embryos reached to 2-cell stage was recorded 24 *hr* after fertilization.

Statistical analysis: All experiments were repeated at least three times. Differences in the proportion of oocyte maturation at each of the meiotic stages (GV, GVBD and MII), degenerated oocytes, fertilization and two cell embryos were statistically analyzed by one way ANOVA using SPSS (version 19) software. An independent-samples t-test was conducted to compare the rates of maturation in each of the meiotic stages (GV, GVBD and MII), degenerated oocytes, fertilization and two cell embryos in ALA treated groups and non ALA treated groups. Percentages were statistically analyzed after arcsine transformation. Assessment of interaction among ALA, presence or absence of cumulus cells and meiotic inhibitors were statistically analyzed by two-way ANOVA. When ANOVA indicated a significant difference ($p<0.05$), Tukey's HSD post hoc was used.

Results

The separate or combined effects of cilostamide and forskolin on nuclear maturation and fertilization of DOs and COCs in the presence or absence of ALA are shown in tables 1 and 2. The total number of retrieved oocytes was 1973. 1077 were COCs and the rest were DOs.

Oocyte nuclear maturation without pretreatment with ALA: A one-way analysis of variance revealed significant differences between the groups in regard to percentage of MII oocytes [(F (7, 16)=45.62, $p<0.05$]. Post hoc comparisons using the Tukey's HSD test revealed that, in one step *in vitro* maturation manner, after an 18 *hr* culture, the rates of MII, in COCs control group (59.51%) were significantly higher (than DOs control group

Table 1. Maturation rates of mouse COCs and DOs following *in vitro* maturation with Cilostamide and Forskolin in the presence or absence of ALA

Groups	n	GV		GVBD		MII		Degeneration	
		ALA+	ALA-	ALA+	ALA-	ALA+	ALA-	ALA+	ALA-
		n/all (%±SD)	n/all (%±SD)	n/all (% ± SD)	n/all (%±SD)	n/all (%±SD)	n/all (%±SD)	n/all (%±SD)	n/all (%±SD)
Control									
COC	352	27/120 (22.50±2.50) ^{a}	54/232 (23.26±0.55) ^{a}	17/120 (14.17±1.44) ^{a}	31/232 (13.34±1.19) ^{a}	73/120 * (60.83±1.44)	138/232 * (59.51±1.59)	3/120 (2.50±2.50)	9/232 (3.88±0.13)
DO	176	29/98 (29.59±2.14) ^{b}	22/78 (28.16±2.07) ^{b}	20/98 (20.40±0.70) ^{b}	16/78 (20.42±1.63) ^{b}	45/98 (45.94±0.64)	33/78 (42.43±2.25)	4/98 (5.19±2.05)	7/78 (8.97±1.38)
Cilostamide									
COC	245	8/120 (6.67±1.44) ^{c}	10/125 (7.96±0.80) ^{c}	35/120 (29.17±1.44) ^{c}	36/125 (28.70±2.08) ^{c}	75/120 (62.50±2.5)	76/125 (60.92±2.73)	2/120 (1.67±1.44)	3/125 (2.41±0.16)
DO	240	15/120 (10.00±2.50) ^{c}	11/120 (9.17±1.44) ^{c}	36/120 (30.00±2.50) ^{c}	35/120 (29.17±1.44) ^{c}	70/120 ** (58.33±1.44)	69/120 ** (57.50±2.50)	2/120 (1.67±1.44)	5/120 (4.17±2.89)
Forskolin									
COC	240	11/120 (9.17±1.44) ^{c}	13/120 (10.67±1.15) ^{c}	29/120 (24.17±3.82) ^{b}	27/120 (22.50±0.00) ^{b}	78/120 (65.00±2.5)	77/120 (64.17±1.44)	2/120 (1.67±1.44)	4/120 (3.33±1.44)
DO	240	14/120 (11.67±1.44) ^{c}	16/120 (13.33 ± 1.44) ^{c}	28/120 (23.33±1.44) ^{b}	27/120 (22.50±2.50) ^{b}	75/120 ** (62.50±2.5)	73/120 ** (60.83±1.44)	3/120 (2.50±2.50)	4/120 (3.33±1.44)
Cilostamide & Forskolin									
COC	240	7/120 (5.83±1.44) ^{c}	8/120 (6.67±1.44) ^{c}	32/120 (25.00±2.50) ^{b}	31/120 (25.83±1.44) ^{b}	82/120 ** (68.33±1.44)	80/120 ** (66.67±1.44)	0/120 (0.00±0.00)	1/120 (0.83±1.44)
DO	240	13/120 (10.83±1.44) ^{c}	14/120 (11.67±1.44) ^{c}	27/120 (22.50±0.00) ^{b}	25/120 (20.83±1.44) ^{b}	79/120 ** (65.83±1.44)	77/120 ** (64.17±1.44)	1/120 (0.83±1.44)	4/120 (3.33±1.44)

Different superscript letters in the same columns indicate significant differences ($p<0.05$).
* indicate significant difference with respective DO groups, ** indicate significant difference with respective control group.
GV: Germinal Vesicle; GVBD: Germinal Vesicle Breackdown; COC: Cumulus Oocytes Complexe; DO: Cumulus Denuded Oocyte; ALA: Alpha Lipoic Acid

Table 2. The rates of fertilization and two cell embryos of mouse COCs and DOs after *in vitro* maturation with meiotic inhibitors in the presence or absence of ALA

Groups	Number of MII		Fertilized/MII (M±SD)		Two Cells/Fertilized (M±SD)	
	ALA-	ALA+	ALA-	ALA+	ALA-	ALA+
Control						
COC	138	73	80/138* (57.95±0.66)	43/73* (58.94±3.29)	57/80* (71.27±4.01)	31/43* (72.06±7.23)
DO	33	45	15/33 (45.37±4.25)	23/45 (51.11±1.92)	8/15 (53.33±5.77)	14/23 (60.45±2.87)
Forskolin						
COC	77	78	48/77 (62.3±2.77)	52/78 (66.68±1.31)	39/48 (81.2±1.2)	46/52 (88.34±6.05)
DO	73	75	38/73** (52.05±2.08)	45/75** (59.95±1.6)	29/38** (76.49±7.06)	35/45** (77.56±5.35)
Cilostamide						
COC	76	75	49/76 (64.41±4.63)	52/75** (69.35±1.42)	40/49 (81.53±1.68)	46/52 (88.45±0.38)
DO	69	70	37/69** (53.62±1.27)	40/70** (57.06±5.19)	28/37** (75.64±1.11)	31/40** (77.31±2.52)
Forskolin+Cilostamide						
COC	80	82	59/80** (73.79±3.28)	64/82** (78.04±3.73)	54/59 (91.49±3.05)	58/64 (90.6±0.52)
DO	77	79	55/77** (71.43±1.98)	57/79** (72.17±4.15)	46/55** (83.62±0.51)	49/57** (86.03±2.5)

* Indicate significant difference with respective DO groups, ** indicate significant difference with respective control group.

COC: Cumulus Oocytes Complexe; DO: Cumulus Denuded Oocyte; ALA: Alpha Lipoic Acid

(42.43%); p<0.05, Table 1). Also, as shown in table 1, the rates of GV (23.26%) and GVBD (13.34%) in COCs control groups were significantly lower than those of DOs control group (28.16% and 20.42% respectively, p<0.05).

In two-step *in vitro* maturation manner, when the COCs were pre-cultured in the medium containing only forskolin and only cilostamide, at the end of cultivation period, no significant difference was observed in the percentages of MII (64.17% and 60.92% respectively;) in comparison with COCs control groups (Table 1), while the MII rate of COCs in medium which contains combination of forskolin-cilostamide (66.67%) was significantly higher than COCs control group (p<0.05, Table 1). The respective rates of MII in DOs groups for cilostamide, forskolin, and combination of cilostamide and forskolin were 57.5%, 60.83%, and 64.17% which were significantly higher (p<0.05) than those of DOs control group, while significantly higher proportion of DOs were matured in the presence of combination of cilostamide and forskolin (p<0.05, Table 1).

The analysis of variance at the end of cultivation period, revealed that the rates of arrested GV oocytes of cultured COCs and DOs in the medium containing forskolin, (10.67% and 13.33% respectively), cilostamide (7.96% and 9.17% respectively) and combination of cilostamide and forskolin (6.67% and 11.67% respectively) were significantly lower than those of respective control group (F (7, 16)=95.96, p<0.05). The means and standard errors are presented in table 1.

Analysis of the rates of GVBD between groups at the end of *in vitro* culture period, revealed significant differences between the groups, [F (7, 16)=30.01, (p<0.05)]. The means and standard errors are presented in table 1. The rates of oocytes arrested at GVBD stage in DOs group with pre-maturation in medium containing forskolin (22.5%) and combination of cilostamide and forskolin (20.63%) were significantly lower (p<0.05) than cilostamide (29.17%) while, there were no significant differences among control DOs, forskolin and combination of cilostamide and forskolin groups. However, these rates in aforementioned treated COCs groups were 22.5%, 28.7% and 25.83%, respectively which were significantly higher than COCs control group.

There were no statistically significant differences between degeneration rates of groups as determined by one-way ANOVA, [F (7, 16)=1.32, p= 0.33].

The rates of fertilization: The means and standard deviation of fertilization and two cell embryo rates are presented in table 2. The analysis of variance revealed significant differences, [F (7, 16)= 33.54 and F (7, 16)=26.59 respectively, ($p<0.05$)]. The rates of fertilization and two cell embryo of MII oocytes in untreated COCs control groups (57.95% and 71.27% respectively), were significantly higher ($p<0.05$) than those of DOs control groups (45.37% and 53.33%, respectively).

The fertilization and two cell embryo rates of COCs groups after pre-maturation with cilostamide (64.41% and 81.53% respectively) and forskolin (62.3% and 81.2% respectively) were similar but they were statistically higher than respective control groups ($p<0.05$) and statistically less than the respective combination of forskolin and cilostamide groups (73.79% and 91.49% respectively; $p<0.05$).

The rates of fertilization and embryos reached to two cell stage of DOs groups after pre-maturation with forskolin were 52.05% and 76.49% respectively and for cilostamide were 53.62% and 75.64% while in the combination of forskolin and cilostamide, they were 71.43% and 83.62% respectively. There was no significant difference between forskolin group and cilostamide group, but they were statistically higher than their respective control groups ($p<0.05$) and were significantly lower than the respective combination of forskolin and cilostamide groups ($p<0.05$).

Effect of ALA on oocyte maturation and fertilization: Comparison of the rates of MII, GV, GVBD, degeneration, fertilization and embryos reached to two cell stage for ALA treated groups and non ALA treated groups revealed no significant differences between the groups (Figure 1).

Interaction between the effects of cumulus cells and meiotic inhibitors on oocyte maturation and fertilization: As shown in figure 1, there were no significant differences in the rates of MII oocytes and fertilization of COCs groups after prematuration with forskolin, cilostamide and combination of forskolin and cilostamide with those of DOs groups, whereas, the rates of MII oocytes, fertilization and two cell embryos of COCs control groups were significantly higher than those of DOs control groups ($p<0.05$).

A two-way ANOVA revealed that there was a significant interaction between the effects of cumulus cells and meiotic inhibitors (forskolin, cilo-

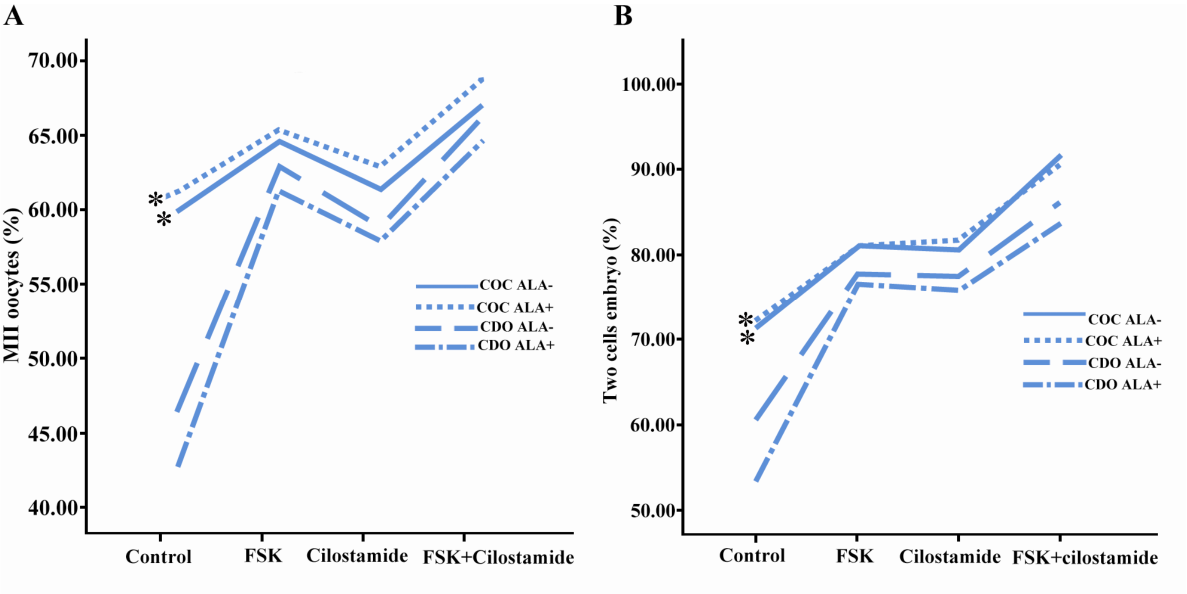

Figure 1. The rates of maturation and two cells embryo under different condition; A: oocyte maturation and B: two cells embryo formation respective treated groups in the presence or absence of ALA and interaction among meiotic inhibitors, ALA and cumulus cells.
* Indicate significant difference with their respective DOs groups.
ALA: Alpha Lipoic Acid, COC: Cumulus Oocyte Complex, DO: Cumulus Denuded Oocyte, FSK: Forskolin, MII: Metaphase II oocyte

stamide and combination of cilostamide and forskolin) on maturation rate, F (3, 40)=32.79, $p<0.05$ and fertilization rate, F (3, 40)=25.07, $p<0.05$. Meiotic inhibitors (forskolin, cilostamide and combination of forskolin and cilostamide) were significantly more effective on the DOs than COCs groups (Figure 1).

Discussion

Despite many advantages, IVM is associated with many challenges. *In vitro* oocyte maturation caused cytoplasmic critical biochemical and molecular events to disappear and led the mature oocyte to be fertilized normally for the formation of the embryo (32). In the oocyte IVM, either nuclear maturation or cytoplasmic maturation is essential (5, 7). In this study, a specific PDE3 inhibitor (cilostamide) and an AC activator (forskolin) have been used in the maturation medium for the concurrent development of oocyte nuclear and cytoplasm maturation. The reversibility process following the cessation of oocyte nuclear maturation using forskolin and cilostamide has been shown by several researchers (14, 25, 27, 28). The results of this study showed that both forskolin and cilostamide did not have any adverse effects on oocyte survival rate, because no significant difference was observed in survival rate of oocytes in different experimental groups. In this regards, Shu and colleagues (28) showed that nuclear maturation was delayed by cilostamide and forskolin, alone or in combination, without negative effects on embryonic development. Elevated cAMP levels of oocyte following the use of PDE3 inhibitors to inhibit oocyte meiotic division was described previously (23, 24, 43). It is known that forskolin can also increase oocyte intracellular levels of cAMP (28). It has been implied that forskolin has complementary and reinforcing effects on the inhibitory activities in oocyte maturation, which is also the basis of the combined treatment of PDE3 inhibitor and AC activator (28).

Several studies have demonstrated the critical role of cumulus cells in the acquisition of developmental competence by oocyte (30, 31, 44) and attributed it to communication between cumulus cells and oocytes via gap junctions (2). In this study, higher rates of maturation and fertilization were observed in one-step *in vitro* maturation manner of COCs without any meiotic inhibitors in comparison with those of DOs, while there were no significant difference between maturation rates and fertilization rates of COCs and DOs in two-step *in vitro* maturation manner with meiotic inhibitors (cilostamide, forskolin and cilostamide plus forskolin). These findings indicate that meiosis inhibitors have a greater impact on the DOs than COCs in two-step *in vitro* culture manner.

Although the maturation rates of COCs obtained in the cilostamide and forskolin groups were similar to the respective control group, their rates of fertilization and two cell embryos were higher than the respective control groups. It seems that in the presence of meiosis inhibitors, delayed activity of PDE, which is essential for the resumption of meiosis (43), can result in the loss of oocytes maturation in the treated groups (27). The role of delayed *in vitro* oocyte maturation in improvement of developmental competence is still controversial. Some studies stated that postponement of *in vitro* oocyte nuclear maturation leads to the failure of developmental competence (45) and others believe that it will lead to improvement of developmental competence (14, 25, 27, 28). In this regards, Vanhoutte and colleagues (27), believed that meiotic inhibitor-induced delay in nuclear maturation results in improved cytoplasmic and nuclear maturation of oocytes.

Nevertheless, in the DOs, the maturation rates of cilostamide and forskolin groups were higher than the respective control groups. One possibility for this finding seems to be related to being exposed to gonadotropin in the maturation medium and the absence of cumulus cells which will accelerate meiotic progression *in vitro* as a non physiological condition of oocyte maturation (27).

Furthermore, forskolin and cilostamide combination was more effective than using them separately. Similar to this result, Shu et al. (28) demonstrated that cilostamide and forskolin have synergistic effect on oocyte maturation, fertilization rate and subsequent embryonic development. In addition, it has been shown that cAMP elevating agents could improve oocyte developmental competence without any adverse effects (14, 25, 26, 29, 46).

Moreover, effects of ALA as a potent antioxidant on the oocyte *in vitro* maturation were examined in the present study. The results showed that there were no significant differences in the rates of maturation, fertilization and two cell embryo formation in the presence or absence of ALA in different groups (one and two step *in vitro* culture manner). Generally, oxidative stress is increased during cultivation (36, 47). Thus, antioxidant supplementation to culture media for reducing oxidative stress seems to be a rational method. However, previous reports on the role of antioxidant in oocyte *in vitro* maturation are very controversial. Meanwhile, conflicting reports of the role of ROS in the oocytes maturation can also exacerbate this situation. In this regard, in another study, the effects of non-enzymatic and enzymatic antioxidant during bovine IVM were assessed and it was shown that cysteine at low glucose concentration significantly improves the developmental competence of oocytes and other extracellular antioxidants did not have any beneficial effects (48). There is also evidence that antioxidant inhibitors could block GV breakdown in COCs and DOs (49). It was also found that oxidative stress induces meiotic arrest (50). In contrast, Tarin et al. (51) showed that ROS not only causes delay in the resumption of meiosis, but also promotes maturation of mouse GV oocytes during IVM.

Additional studies have examined the effects of antioxidants on oocyte *in vitro* maturation. It has been shown that, by adding cysteamine, cysteine, and β-mercaptoethanol to maturation medium during cultivation period of porcine oocytes, the rate of embryo production could be improved (52, 53), while adding β-mercaptoethanol, superoxide dismutase, or ascorbic acid to the bovine oocytes maturation medium had no positive effect on subsequent development (54).

It has been demonstrated that ALA improves developmental competence of cultured mouse pre-antral follicles through reduction of ROS production and enhancement of total anti-oxidant capacity (38), but in the present study, the presence of ALA in the GV oocyte maturation medium in one or two step culture manner did not have any positive effect on oocytes maturation and fertilization. This contradiction seems to be related to the cultivation period. It has been confirmed that longer cultivation period results in increased ROS production (38, 55). Talebi and colleagues (38), have cultured pre-antral follicles for 12 days, whereas in the present study, the maximum duration of *in vitro* oocyte culture was 36 *hr*. In this regard, it is demonstrated that *in vitro* oocyte maturation does not lead to increased ROS generation in both COCs and DOs (56). Furthermore, it seems that this is due to oocyte's ability to express genes encoding anti-oxidant enzymes as shown in mouse and human oocytes (57) as well as in early murine and bovine embryos (58). The antioxidant activity of COC was attributed to the cumulus cells, however, oocytes have their own mechanisms to avoid destructive effects of ROS (56, 58). This explains the inability of the antioxidant to improve maturation and fertilization of both COCs and DOs after *in vitro* maturation.

Conclusion

In conclusion, the use of combination of forskolin and cilostamide in GV oocyte maturation medium was more effective than the use of each of them as the only meiotic inhibitor in two step *in vitro* maturation manner. Furthermore, forskolin and cilostamide alone or in combination were more effective on DOs than COCs., The results of this study, for the first time, demonstrated that supplementation of ALA to mouse GV oocyte maturation medium could not improve maturation and fertilization of both COCs and DOs in one or two step *in vitro* maturation manner.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Canipari R. Oocyte--granulosa cell interactions. Hum Reprod Update. 2000;6(3):279-89.
2. Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol Reprod Dev. 2002;61(3):414-24.
3. Combelles CM, Carabatsos MJ, Kumar TR, Matzuk MM, Albertini DF. Hormonal control of somatic cell oocyte interactions during ovarian follicle development. Mol Reprod Dev. 2004;69(3):347-55.
4. Gilchrist RB, Ritter LJ, Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. Anim Reprod Sci. 2004;82-83:431-46.
5. Smith GD. In vitro maturation of oocytes. Curr Womens Health Rep. 2001;1(2):143-51.
6. Trounson A, Anderiesz C, Jones G. Maturation of human oocytes in vitro and their developmental competence. Reproduction. 2001;121(1):51-75.

7. Ali A, Benkhalifa M, Miron P. In-vitro maturation of oocytes: biological aspects. Reprod Biomed Online. 2006;13(3):437-46.
8. Salamone DF, Damiani P, Fissore RA, Robl JM, Duby RT. Biochemical and developmental evidence that ooplasmic maturation of prepubertal bovine oocytes is compromised. Biol Reprod. 2001;64(6): 1761-8.
9. Schramm RD, Paprocki AM, VandeVoort CA. Causes of developmental failure of in-vitro matured rhesus monkey oocytes: impairments in embryonic genome activation. Hum Reprod. 2003;18(4):826-33.
10. Jimenez-Macedo AR, Izquierdo D, Urdaneta A, Anguita B, Paramio MT. Effect of roscovitine on nuclear maturation, MPF and MAP kinase activity and embryo development of prepubertal goat oocytes. Theriogenology. 2006;65(9):1769-82.
11. Combelles CM, Cekleniak NA, Racowsky C, Albertini DF. Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes. Hum Reprod. 2002;17(4):1006-16.
12. Eppig JJ, Schultz RM, O'Brien M, Chesnel F. Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes. Dev Biol. 1994;164(1):1-9.
13. Anderiesz C, Fong CY, Bongso A, Trounson AO. Regulation of human and mouse oocyte maturation in vitro with 6-dimethylaminopurine. Hum Reprod. 2000;15(2):379-88.
14. Nogueira D, Cortvrindt R, De Matos DG, Vanhoutte L, Smitz J. Effect of phosphodiesterase type 3 inhibitor on developmental competence of immature mouse oocytes in vitro. Biol Reprod. 2003;69 (6):2045-52.
15. Nogueira D, Cortvrindt R, Everaerdt B, Smitz J. Effects of long-term in vitro exposure to phosphodiesterase type-3 inhibitors on follicle and oocyte development. Reproduction. 2005;130(2):177-86.
16. Dieleman SJ, Hendriksen PJ, Viuff D, Thomsen PD, Hyttel P, Knijn HM, et al. Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre implantation embryos. Theriogenology. 2002;57(1):5-20.
17. Sela-Abramovich S, Edry I, Galiani D, Nevo N, Dekel N. Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation. Endocrinology. 2006;147(5):2280-6.
18. Bilodeau-Goeseels S. Effects of phosphodiesterase inhibitors on spontaneous nuclear maturation and cAMP concentrations in bovine oocytes. Theriogenology. 2003;60(9):1679-90.
19. Masciarelli S, Horner K, Liu C, Park SH, Hinckley M, Hockman S, et al. Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility. J Clin Invest. 2004;114(2):196-205.
20. Conti M, Andersen CB, Richard F, Mehats C, Chun SY, Horner K, et al. Role of cyclic nucleotide signaling in oocyte maturation. Mol Cell Endocrinol. 2002;187(1-2):153-9.
21. Horner K, Livera G, Hinckley M, Trinh K, Storm D, Conti M. Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. Dev Biol. 2003;258(2):385-96.
22. Soderling SH, Beavo JA. Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. Curr Opin Cell Biol. 2000;12(2):174-9.
23. Sasseville M, Cote N, Guillemette C, Richard FJ. New insight into the role of phosphodiesterase 3A in porcine oocyte maturation. BMC Dev Biol. 2006;6:47.
24. Nogueira D, Albano C, Adriaenssens T, Cortvrindt R, Bourgain C, Devroey P, et al. Human oocytes reversibly arrested in prophase I by phosphodiesterase type 3 inhibitor in vitro. Biol Reprod. 2003;69(3):1042-52.
25. Nogueira D, Ron-El R, Friedler S, Schachter M, Raziel A, Cortvrindt R, et al. Meiotic arrest in vitro by phosphodiesterase 3-inhibitor enhances maturation capacity of human oocytes and allows subsequent embryonic development. Biol Reprod. 2006;74(1):177-84.
26. Bagg MA, Nottle MB, Grupen CG, Armstrong DT. Effect of dibutyryl cAMP on the cAMP content, meiotic progression, and developmental potential of in vitro matured pre-pubertal and adult pig oocytes. Mol Reprod Dev. 2006;73(10):1326-32.
27. Vanhoutte L, De Sutter P, Nogueira D, Gerris J, Dhont M, Van der Elst J. Nuclear and cytoplasmic maturation of in vitro matured human oocytes after temporary nuclear arrest by phosphodiesterase 3-inhibitor. Hum Reprod. 2007;22(5):1239-46.
28. Shu YM, Zeng HT, Ren Z, Zhuang GL, Liang XY, Shen HW, et al. Effects of cilostamide and forskolin on the meiotic resumption and embryonic development of immature human oocytes. Hum Reprod. 2008;23(3):504-13.
29. Luciano AM, Modina S, Vassena R, Milanesi E, Lauria A, Gandolfi F. Role of intracellular cyclic adenosine 3',5'-monophosphate concentration and oocyte-cumulus cells communications on the acquisition of the developmental competence during in vitro maturation of bovine oocyte. Biol Reprod. 2004;70(2):465-72.
30. Zavareh S, Saberivand A, Salehnia M. The effect of progesterone on the in vitro maturation and developmental competence of mouse germinal vesicle oocytes. Int J Fertil Steril. 2009;3(1):21-8.

31. Zavareh S, Salehnia M, Saberivand A. Comparison of different vitrification procedures on developmental competence of mouse germinal vesicle oocytes in the presence or absence of cumulus cells. Int J Fertil Steril. 2009;3(3):111-8.

32. Gilchrist RB, Thompson JG. Oocyte maturation: emerging concepts and technologies to improve developmental potential in vitro. Theriogenology. 2007;67(1):6-15.

33. Jamnongjit M, Hammes SR. Oocyte maturation: the coming of age of a germ cell. Semin Reprod Med. 2005;23(3):234-41.

34. Norris RP, Ratzan WJ, Freudzon M, Mehlmann LM, Krall J, Movsesian MA, et al. Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. Development. 2009;136(11):1869-78.

35. Norris RP, Freudzon M, Mehlmann LM, Cowan AE, Simon AM, Paul DL, et al. Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. Development. 2008;135(19):3229-38.

36. Combelles CM, Gupta S, Agarwal A. Could oxidative stress influence the in-vitro maturation of oocytes? Reprod Biomed Online. 2009;18(6):864-80.

37. Agarwal A, Gupta S, Sikka S. The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol. 2006;18(3):325-32.

38. Talebi A, Zavareh S, Kashani MH, Lashgarbluki T, Karimi I. The effect of alpha lipoic acid on the developmental competence of mouse isolated preantral follicles. J Assist Reprod Genet. 2012;29(2):175-83.

39. Yamasaki M, Kawabe A, Nishimoto K, Madhyastha H, Sakakibara Y, Suiko M, et al. Dihydro-alpha-lipoic acid has more potent cytotoxicity than alpha-lipoic acid. In Vitro Cell Dev Biol Anim. 2009;45(5-6):275-80.

40. Packer L, Witt EH, Tritschler HJ. alpha-Lipoic acid as a biological antioxidant. Free Radic Biol Med. 1995;19(2):227-50.

41. Azadbakht M, Valojerdi MR. Development of vitrified-warmed mouse embryos co-cultured with polarized or non-polarized uterine epithelial cells using sequential culture media. J Assist Reprod Genet. 2008;25(6):251-61.

42. Summers MC, McGinnis LK, Lawitts JA, Raffin M, Biggers JD. IVF of mouse ova in a simplex optimized medium supplemented with amino acids. Hum Reprod. 2000;15(8):1791-801.

43. Richard FJ, Tsafriri A, Conti M. Role of phosphodiesterase type 3A in rat oocyte maturation. Biol Reprod. 2001;65(5):1444-51.

44. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update. 2008;14(2):159-77.

45. Son WY, Lee SY, Lim JH. Fertilization, cleavage and blastocyst development according to the maturation timing of oocytes in in vitro maturation cycles. Hum Reprod. 2005;20(11):3204-7.

46. Thomas RE, Thompson JG, Armstrong DT, Gilchrist RB. Effect of specific phosphodiesterase isoenzyme inhibitors during in vitro maturation of bovine oocytes on meiotic and developmental capacity. Biol Reprod. 2004;71(4):1142-9.

47. Luvoni GC, Keskintepe L, Brackett BG. Improvement in bovine embryo production in vitro by glutathione-containing culture media. Mol Reprod Dev. 1996;43(4):437-43.

48. Ali AA, Bilodeau JF, Sirard MA. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. Theriogenology. 2003;59(3-4):939-49.

49. Takami M, Preston SL, Toyloy VA, Behrman HR. Antioxidants reversibly inhibit the spontaneous resumption of meiosis. Am J Physiol. 1999;276(4 Pt 1):E684-8.

50. Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, et al. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. J Pineal Res. 2008;44(3):280-7.

51. Tarin JJ. Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. Mol Hum Reprod. 1996;2(10):717-24.

52. de Matos DG, Furnus CC, Moses DF, Baldassarre H. Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured in vitro. Mol Reprod Dev. 1995;42(4):432-6.

53. de Matos DG, Furnus CC, Moses DF, Martinez AG, Matkovic M. Stimulation of glutathione synthesis of in vitro matured bovine oocytes and its effect on embryo development and freezability. Mol Reprod Dev. 1996;45(4):451-7.

54. Blondin P, Coenen K, Sirard MA. The impact of reactive oxygen species on bovine sperm fertilizing ability and oocyte maturation. J Androl. 1997;18(4):454-60.

55. Abedelahi A, Salehnia M, Allameh AA, Davoodi D. Sodium selenite improves the in vitro follicular development by reducing the reactive oxygen species level and increasing the total antioxidant

capacity and glutathione peroxide activity. Hum Reprod. 2010;25(4):977-85.

56. Cetica PD, Pintos LN, Dalvit GC, Beconi MT. Antioxidant enzyme activity and oxidative stress in bovine oocyte in vitro maturation. IUBMB Life. 2001;51(1):57-64.
57. El Mouatassim S, Guerin P, Menezo Y. Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. Mol Hum Reprod. 1999;5(8):720-5.
58. Harvey MB, Arcellana-Panlilio MY, Zhang X, Schultz GA, Watson AJ. Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary bovine oviduct cultures employed for embryo coculture. Biol Reprod. 1995;53(3):532-40.