

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-test and 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.

Keywords: ALA, cAMP-elevating agents, Cumulus cell, *In vitro* maturation, Mouse, Oocyte.

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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 developmental 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, peroxy 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 Milli-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 μ M cilostamide, (III) IVM with 50 μ M 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 TCM₁₉₉ 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 $\mu\text{g/ml}$ 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 TCM₁₉₉ 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 TCM₁₉₉ supplemented with 0.22 g/L NaHCO₃, 100 IU/ml penicillin and 75

$\mu\text{g/ml}$ 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 dimethylsulphoxide (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% CO₂ 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% CO₂ 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 $1-2 \times 10^6/\text{ml}$ 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 oo-

cytes and spermatozoae were incubated for 6 hr at 37°C in 5% CO₂ 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% CO₂ 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 statically 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 Breakdown; COC: Cumulus Oocytes Complex; 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/MIH (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 Complex; 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 pre-maturation 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-

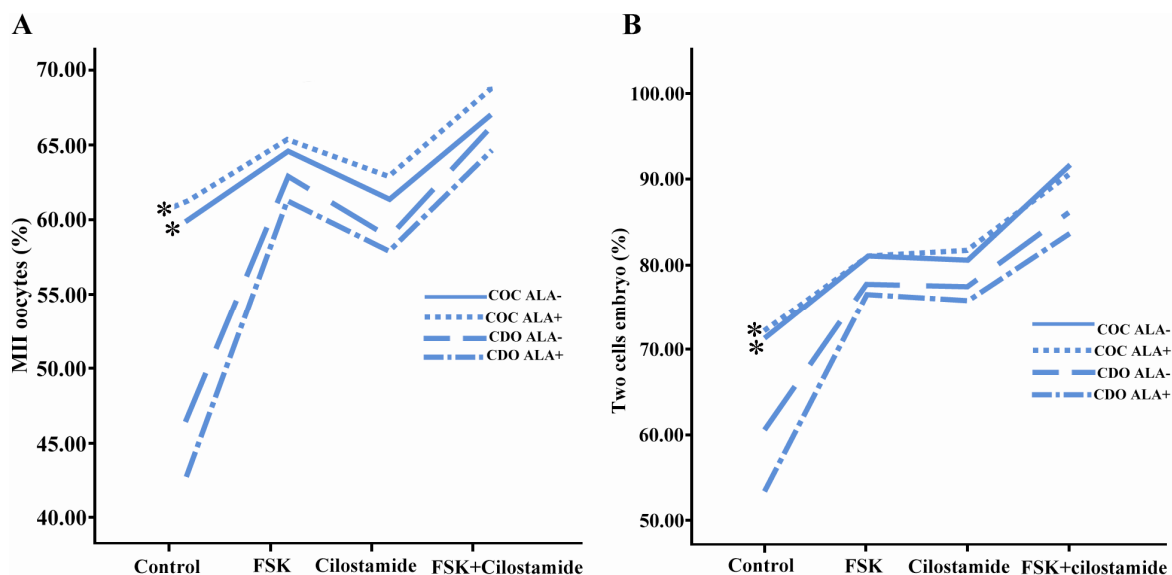


Figure 1. The rates of maturation and two cells embryo under different condition; A: oocyte maturation and B: two cells embryo formation respective treated groups in the presence or absence of ALA and interaction among meiotic inhibitors, ALA and cumulus cells. * Indicate significant difference with 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 sup-

plementation 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.

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