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GAMETE BIOLOGY

Effects of retinoic acid on maturation of immature mouse oocytes in the presence and absence of a granulosa cell co-culture system

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Abstract
Purpose Evaluation of the all-trans retinoic acid (t-RA) effects on in vitro maturation (IVM) and in vitro fertilization (IVF) of immature mouse oocytes in the presence and absence of granulosa cell monolayer.
Methods Denuded oocytes isolated from mice ovaries and matured in IVM medium alone (Control I), IVM medium in the presence of granulosa cells (Control II), IVM medium with t-RA (Experimental I) and IVM medium simultaneously with t-RA and granulosa cells (Experimental II). After 24 h, matured oocytes were fertilized in T6 medium and their development was followed until the blastocyst stage. Metaphase II oocytes ploidy were evaluated by chromosome counting.
Results The t-RA group compared to the control groups showed no obvious abnormalities. Additionally maturation and embryo development rates significantly increased in the t-RA treated granulosa cell co-culture system.
Conclusions In conclusion, association of t-RA with granulosa cell co-culture during in vitro maturation increases meiosis resumption, formation of metaphase II oocytes, as well as 2-cell and blastocyst stage embryos.

Keywords In vitro maturation · Granulosa cells · Immature oocytes · Retinoic acid co- culture

Capsule Beneficial effects of retinoic acid are described for denuded mouse oocytes matured in co-culture with granulosa cells.

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Introduction

Many IVM studies have been performed in different mammalian species such as pigs and cattle [1]; this technique is used for the commercial production of laboratory animal embryos such as rats and mice [2]. Although this technique is successful in animals, in human there has been less success [3]. Females diagnosed with disorders such as ovarian failure and polycystic ovarian syndrome (PCOS) have a low ovarian stimulation response and the use of reproductive technologies for such disorders or for in vitro maturation of immature oocytes causes problem with the maturation of oocytes in addition to decreasing their developmental competence [2, 4]. Therefore, improving IVM protocols by adding different hormones, growth factors [5], and vitamins [6, 7] is necessary in order to increase medium efficiency to support the competence of immature oocytes for fertilization and embryogenesis [8]. Retinoic acid (RA) is an important metabolite and component of vitamin A (all-trans-retinol) which is necessary for human reproduction [9]. RA can be found in naturals and synthetic compositions [6] and is considered to be an important modifier of vertebrate development, cell differentiation and tissue function [10].

Different types of RA are important in the formation of vertebrate embryos, however RA alteration in statement of homeobox genes, growth factors, and their receptors can be

traced on cytoplasmic maturation and oocytes potency for developmental progress [11]. In 1999, it was shown that retinol improved in vitro embryo viability in sheep [12]. Some years later, it has been proven that the addition of RA during the IVM procedure improves early development of bovine oocytes [7]. According to Alminana, the use of RA in the maturation process increased embryo development in porcine oocytes [13]. On the other hand, studies have shown the usefulness of the co-culture with a monolayer of various somatic cell types on both in vitro oocyte maturation and mammalian embryonic development [2, 14–17]. Different types of somatic cells have been routinely used for co-culture systems including oviduct and uterine cells, fetal bovine uterine fibroblasts, African green monkey kidney epithelial cells (Vero cells) [17, 18] and granulosa cells [19]. In another research, Duque et al. have demonstrated that supplementation of culture medium with RA during the maturation period of bovine cumulus-oocyte complexes, in the presence of roscovitine, improved cytoplasmic maturation [5]. In this manner, Mohan et al. have shown that cumulus granulosa cells contain endogenously activated retinoid receptors that can reply to RA [19]. The beneficial effects of RA on in vitro maturation of cumulus oocyte complexes has been previously described [5, 6, 12, 20, 21] however no reports have discussed the effects of RA on the developmental competence of naked immature oocytes. In a previous study, we have shown that the use of a physiological dose of all-trans retinoic acid (t-RA; 2 μM) during in vitro maturation and under precise conditions could improve both maturation and developmental competence of denuded immature mouse oocytes [22]. Therefore, based on this and other studies [5, 6, 12, 19–22] we assume that the cumulus granulosa cells might be a target for added RA during in vitro maturation, which could improve embryo development. In this study, we evaluate the effects of t-RA in association with a co-culture granulosa cell monolayer on the IVM of naked immature mice oocytes and then follow them until the blastocyst stage.

Materials and methods

All chemicals were purchased from Sigma (St Louis, MO, USA) except follitropin alfa (Gonal-F), recombinant human follicle stimulating hormone (rhFSH), and human chorionic gonadotropin (hCG) which were purchased from Organon (Oss, Netherlands), and fetal calf serum (FCS) which was purchased from Invitrogen (Carlsbad, CA, USA).

Granulosa cell preparation and culture

The 6–8 weeks old female NMRI mice were synchronized and super-ovulated by intraperitoneal injections (i.p.) of 7.5 IU pregnant mare serum gonadotropin (PMSG) followed 48 h later by 7.5 IU of hCG. Their oviducts were removed 14–16 h after hCG injection and placed in T6 medium supplemented with 4 mg/ml bovine serum albumin (BSA). Then granulosa oocyte complexes (GOC) were collected and five GOCs were placed into a 4-well Nunclon dish and cultured in 500 μl of Dulbecco's modified eagle's medium (DMEM) supplemented with 15% FCS. After 72–96 h, unattached cells and dead oocytes were removed by exchanging the previous medium with fresh medium [15].

Collection of immature oocytes

Animals were purchased from Razi Institute, Karaj (Iran). Female mice (6–8 weeks-old) were killed by cervical dislocation. Detached ovaries were dissected in alpha modification of minimum essential medium (α-MEM) supplemented with 5% FCS. Denuded and intact immature oocytes at the germinal vesicle (GV) stage were collected for in vitro maturation and fertilization by stereomicroscope.

Experimental design

A total of 2258 naked oocytes with a mean diameter of 60–65 μm were randomly assigned to each of the sham, control and experimental groups. As ethanol (v/v) was used as a solvent for t-RA, therefore in the Sham group 0.2% v/v of ethanol was added to the maturation medium. In the Control I group, neither t-RA nor granulosa cell co-culture was used. In the Control II group, oocytes were cultured on a monolayer of granulosa cells without t-RA. In addition, in the experimental groups, oocytes were exposed to the best dose (2 μM) of t-RA [22] in the presence (Experimental II group) and absence (Experimental I group) of granulosa cell co-cultures respectively.

In vitro maturation of GV oocytes

Matuartion medium consisted of α-MEM medium supplemented with 6 mg/ml penicillin, 5 mg/ml streptomycin, 100 mIU/ml rhFSH, 7.5 IU/ml hCG and 5% FCS. After 24 h of culture in different conditions, according to the abovementioned groups, the maturity of the oocytes was assessed by an inverted microscope. Oocytes were classified as GV, germinal vesicle breakdown (GVBD) and metaphase II (MII).

Chromosome count

MII oocytes in the Experimental I and Control I groups were placed in tyrode acid solution for 5–10 s. Then, oocytes were placed in a 1% sodium citrate, hypotonic solution, for 10–15 min after which MII oocytes were

placed individually on clean glass slides with a small amount of methanol/acetic acid (3:1) solution as a fixative. Slides were air-dried and stained with 4% giemsa for 5 min. The ploidy of the MII oocytes was examined by counting the number of chromosomes [23].

In vitro fertilization of IVM oocytes

In vitro fertilization was performed with the spermatozoa retrieved from the caudate of the epididymis of NMRI male mice. Spermatozoa were dispersed in T6 medium with 15 mg/ml BSA and subsequently diluted to a final concentration of 1×10^6 sperm/ml and incubated for at least 90 min within a humidified 5% CO_2 incubator at 37°C in order to allow capcitation. MII oocytes were inseminated by transfer to the sperm droplets, and washed 4–5 h after insemination. The percentage of 2 pre-nucleus (PN) formations was recorded as the fertilization rate. Next oocytes were cultured in groups of 5–10 oocytes per 50 µl drop of T6 medium (without RA) supplemented with 4 mg/ml BSA at 37°C and incubated in a 5% CO_2 incubator [24].

Embryo development

Fertilization was assessed 24 h after insemination on the third day following fertilization, according to the cleavage rate of the two-cell stage embryos. Compaction of the embryos was assessed and their blastocyst rates were considered on days 4–5.

Statistical analysis

GV, GVBD, MII, embryo development and blastocyst data were considered as categorical variables. ANOVA tests for independence and trend were used to determine statistically significant differences. All experiments were replicated 11–15 times. Probabilities of $p<0.001$, $p<0.01$and $p<0.05$ were considered to be statistically different between the control and experimental groups.

Results

In vitro maturation

Oocytes meiotic competency among the different groups was determined after 24 h of in vitro maturation. As shown in Table 1 the mean proportion of MII oocytes in the Experimental II group was 79.6%, which was significantly higher than the Control I and Sham groups ($p<0.001$) and the Control II group ($p<0.01$). Additionally, the mean proportion of GV stage oocytes was 24.4%, 15.4%, 24.3%, 11.0% and 6.2% in the Control I, Control II, Sham, Experimental I and Experimental II groups respectively. A significant difference existed between different groups regarding the percentage of GV stage oocytes.

Oocytes chromosomal counts

A total of 68 oocytes at the MII stage were examined in the Experimental I group ($n=36$) and Control I ($n=32$) groups. The percentages of normal counts of the chromosomes were 94.4% and 87.5% in the Experimental I and Control I groups respectively. These data by chi-square demonstrate that 2 µM t-RA in comparison with the Control I group showed no significant difference.

In vitro fertilization

Table 2 shows the developmental competence of mouse embryos. The mean proportion of two-cell stage embryos was higher in the Experimental II group (62.5%) compared to the Control I (53.5%) and Control II (57.7%) groups. The rate of two-cell stage embryos in the Sham group was significantly lower than both Experimental I and II groups ($p<0.05$). The mean proportion of embryos which reached the blastocyst stage was higher in the Experimental II group (28.4%) in comparison with the Control I (12.3%), Control II (16.5%), Sham (9.4%) and Experimental I (22%) groups. The rate of embryos that developed to the blastocyst stage

Table 1 Comparison of in vitro maturation in the control, sham and experimental groups

Experimental Groups	Oocytes number	Retinoicacid (µM)	Ethanol (v/v)	G	R	GV number (% + SE)	GVBD number (% + SE)	MII number (% + SE)
Control I	469	–	–	–	15	111 (24.4±2.1)$^{a\alpha}$	70 (16.0±2.3)	288 (60.0±2.4)$^{\beta}$
Experimental I	459	2	0.2	–	15	50 (11.0±1.4) $^{\beta}$	60 (13.2±2.0)	349 (75.9±2.3)
Control II	451	–	–	+	11	69 (15.4±2.8) ^{b}	88 (21.1±3.3)	294 (63.4±3.9) ^{y}
Experimental II	444	2	0.2	+	11	26 (6.2±1.3)$^{\beta}$	62 (14.2±1.6)	356(79.6±2.4)$^{\alpha x}$
Sham	435	–	0.2	–	15	109 (24.3±2.4) $^{a\alpha}$	70 (16.6±2.0)	256 (59.0±2.2)$^{\beta}$

G Granulosa co-culture, *R* number of replicates in group, *GV* Germinal vesicle, *GVBD* Germinal vesicle breakdown, *MII* Metaphase II. Data express ANOVA results as mean percentage ± SE. Values with different superscripts are significantly different. a,b ($p<0.05$); x,y ($p<0.01$); α, β ($p<0.001$)

Table 2 Comparison of embryonic development of in vitro matured mouse oocytes between the control, sham and experimental groups

Condition during IVM				Embryonic Stage			
Groups	t- RA (μM)	EtOH (V/V)	G	R	2 PN number (% ± SE)	2-cell number (% ± SE)	Blastocyst number (% ± SE)
Control I	–	–	–	15	210 (72.4±5.6)^{b}	102 (53.5±6.0)	22 (12.3±1.4) $^{\beta}$
Experimental I	2	0.2	–	15	214 (60.5±5.1)	134 (61.7±3.2)^{a}	44 (22.0±1.7)
Control II	–	–	+	11	164 (60.0±4.7)	89 (57.7±5.7)	24 (16.5±2.3) ^{y}
Experimental II	2	0.2	+	11	184 (51.0±1.7) ^{ay}	113(62.5±2.6)^{a}	48 (28.4±2.4) $^{x\alpha}$
Sham	–	0.2	–	15	202 (77.8±5.0) ^{x}	85 (42.2±5.1)^{b}	18 (9.4±1.4) $^{\beta}$

t-RA all-trans retinoic acid, *EtOH* ethanol, *G* Granulosa co-culture, *R* number of replicates in group. *PN* pre nucleus. The mean percentage of embryonic development (2 cell and blastocyst) is expressed according to 2 pre nucleus (PN) numbers. ANOVA test values with different superscripts are significantly different:a,b ($p<0.05$); x,y ($p<0.01$); α,β ($p<0.001$)

after 120 h was significantly increased in the Experimental II group compared to the Control II ($p<0.01$) and the Sham and Control I ($p<0.001$) groups.

Discussion

The beneficial effects of retinol metabolites on in vitro cytoplasmic maturation and embryo development have been previously described [5, 6, 12, 20, 25] however there are no reports about the simultaneous effects of t-RA and co-culture systems of granulosa cells on the developmental potency of cumulus-free immature oocytes. Following our previous observation [22], in the present study we have shown that the use of 2 μM t-RA in maturation medium with granulosa cell monolayer improves developmental competence of naked immature mice oocytes.

Oocyte maturation requires nuclear and cytoplasmic maturation. The amount of stored mRNA, proteins and increased cyclic adenosine monophosphate (cAMP) levels are reduced within the cytoplasm when cytoplasm maturation proceeds [26]. At this time meiosis resumption starts. Increased or maintained high levels of cAMP in oocytes prevent the meiosis resumption [27]. Bagavandoss and Midgeley [28] have suggested that RA may stimulate FSH for the induction of LH receptors and also encourage progesterone production and decrease cAMP levels. Cortical granule migration as a maturation clue could be affected by RA since RA improves granular migration [29, 30]. Expression of several antioxidant enzymes during in vitro oocyte maturation activates several internal defense mechanisms that protect oocytes against reactive oxygen species (ROS) [25]. It has been demonstrated that antioxidants increase in culture and maturation medium or in the embryo culture in a reduced O_2 atmosphere could assist to in vitro survival of embryos in a variety of species [31, 32]. A number of studies have shown that retinoids participate in a biological anti-oxidant network and have been implicated as important regulators of redox signaling pathways [33, 34]. Retinol derivatives can quench oxygen molecules and interact with other antioxidant compounds [35]. It has been shown that RA could protect oocyte against oxidative stress that has been induced by apoptosis [36, 37]. These anti-apoptotic effects of RA are mediated by nuclear receptors [19]. The majority of RA nuclear receptors, including RA receptor alpha (RARα) and β, retinoid X receptor-α (RXRα) and β, retinaldehyde dehydrogenase and peroxisome-proliferators have been detected in porcine oocytes [12], bovine oocytes and also in cumulus cells [38]. Apart from the oocyte, the t-RA influence could be exerted through the granulosa cell monolayer [5]. Chin et al. [39] have shown that granulosa cells in a mouse growing follicle have a remarkable role in oocyte maturation and therefore are suitable candidates for co-culture. Granulosa cells synthesize RA from t-RA and the presence of RARα demonstrates that these cells are targets of RA in rats, humans and mice [40, 41]. RA by exerting its effects directly on the oocyte or adjacent cumulus-granulosa cells or through both autocrine and paracrine manner could modify the transcriptional activity within the cumulus-oocyte complex [38]. The effects of 0.25 μM, 0.5 μM, 1 μM, 2 μM, 5 μM and 10 μM concentrations of t-RA doses on oocytes maturation have been previously investigated [22]. Mouse oocytes which exposed to 2 μM concentrations of t-RA have shown subsequent blastocyst development. Experimental groups under 5 and 10 μM treatment of RA were excluded from further study due to the reduced maturation rate in these groups. In support of the obtained results with the use of high doses of RA (100 μM), in vitro maturation of bovine oocytes has been confirmed to be harmful [9]. Moreover, in a study by Gomez [20], it has been concluded that the harmful effect of high RA doses may result from a reaction between RA and follicle-stimulating hormone (FSH). Therefore, following study selected 2 μM of t-RA as the best dose for oocytes maturation [22]. In this study we investigated the effects of 2 μM t-RA in association with co-culture granulosa cell monolayer on 2258 naked immature oocytes

from mice. As shown in our results, the numbers of oocytes that matured in the culture medium containing RA and co-culture increased in comparison with the control groups. In fact, oocytes that matured in the absence of t-RA and granulosa cell co-culture showed lower developmental rates to the blastocyst stage. We have demonstrated that granulosa co-culture has a beneficial effect on oocytes maturation and in vitro embryonic development as shown by Anderiesz [42] but it does not significantly change MII percentage and blastocyst rate. Absence of the junction between oocytes and granulosa cells in the granulosa cells co- culture group could be an acceptable explanation for the lesser effects of this group on oocytes maturation. However, addition of RA to the granulosa co-culture could improve the maturation condition. This is probably arbitrated through a regulatory role on the microenvironment surrounding the immature oocytes by excretion of various meiosis-promoting factors within the culture medium [5, 43, 44]. Our results support the assumption that t-RA and granulosa cell co-cultures are two factors that enhance oocytes maturation ability. This research has attempted to create an appropriate competent murine model for in vitro oocyte meiotic maturation. Additionally, it could be better suited for developing IVM protocols as a clinical aid for human reproduction. Finally, the use of t-RA, when associated with granulosa cell monolayer can increase maturation of immature oocytes and improve the development of resultant mice embryos. Further studies should be undertaken to clarify the mechanisms involved in t-RA and maturation of oocytes in the presence of the co-culture of granulosa cells.

In conclusion, this study has demonstrated that addition of t-RA to the co-culture system of cumulus-free oocytes with monolayer of granulosa cells during IVM improves its subsequent development in mice.

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