The effect of all-trans retinol on in vitro mouse embryo's developmental competence

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Abstract:

BACKGROUND: All-trans retinol is a biological antioxidant scavenging the ROS in the cell culture. **OBJECTIVES:** This study was conducted to investigate the effect of all-trans retinol in fertilization and culture medium on mouse embryo's developmental competence. **METHODS:** This study was designed into two experiments. In the first experiment, in vitro mature oocytes were co-cultured with sperm in fertilization medium containing different concentrations of all-trans retinol (0, 1, 5, and 10 µM). After fertilization, zygotes in each group were separately cultured in CZB culture medium for 5 days to the blastocyst stage. In the second experiment, in vitro produced zygotes were cultured in CZB culture medium containing different concentrations of all-trans retinol (0, 1, 5, and 10 µM) for 5 days to the blastocyst stage. **RESULTS:** In the first experiment, the blastocyst formation rate significantly increased by 5 µM in all-trans retinol, which was more than those of the other groups. Also, percentage of grade one embryos was significantly higher in the presence of 5 µM all-trans retinol than those in the presence of 0 and 1 µM all-trans retinol. In the second experiment, different concentrations of all-trans retinol could not alter blastocyst formation rate; however, the percentage of grade one embryo was higher in the presence of 10 µM all-trans retinol than that of the control group. **CONCLUSIONS:** These results showed that supplementation of fertilization medium with 5 µM all-trans retinol could improve mouse embryo's development and morphology. On the other hand, supplementation of embryo culture medium can improve mouse embryo morphology without any effect on embryo developmental competence.

Introduction

Retinoids have an important role on oocyte maturation and embryonic development. Differentiation induced by retinoid is via specific changes in the expression of some genes (Brown et al., 2003). Administration of retinol or ß-carotene has resulted in increased embryo viability in rabbits and rats (Besenfelder et al., 1993; Wellik and DeLuca, 1995). Retinoic acid may promote cytoplasmic maturation of bovine oocytes via its effect on some gene expressions including gonadotrophin receptors, COX-2, and NOS in cumulus-granulosa cells (Ikeda

et al., 2004). Addition of retinol to the maturation medium may improve embryonic development of bovine oocytes which is indicated by their increased blastocyst rate (Livingstone et al., 2005). Also, addition of retinol to the in vitro maturation (IVM) medium prevented heat-induced reductions in the development of bovine oocytes to blastocyst stage (Lawrence et al., 2004). It has been shown that the addition of 9-cis-retinoic acid to IVM medium affects trophectoderm differentiation, total cell number, and inner cell mass to trophoblast cell ratios in cattle (Gomez et al., 2004; Hidalgo et al., 2003). The mechanism by which retinol affects oocyte maturation and embryonic development is not known. Retinoic acid may have an effect on oocyte maturation through its effects on FSH or LH receptor expression in granulosa cells (Hattori et al., 2000; Minegishi et al., 2000). In addition, retinoid may promote development by an endogenous oxidative-stress protection mechanism (Guerin et al., 2001). It has been well established that the improvement of in vitro fertilization (IVF) condition results in the increase of embryo development. Like in vitro culture, it seems that in vitro fertilization of matured oocytes at high concentration of O2 results in the increase of the production of reactive oxygen species (ROS) (Luvoni et al., 1996). On the other hand, it has been reported that the presence of ROS impairs blastocyst development during IVF (Torok et al., 2002) and in vitro culture (IVC) in mice (Bedaiwy et al., 2002). Then, one of the approaches for improving mouse embryo development is the supplementation of fertilization and culture media with anti-oxidant reagents like all-trans retinol. Therefore, the aims of this study were to investigate a) the effect of all-trans retinol during IVF and b) the effect of IVC on mouse embryo developmental competence and morphology.

Materials and Methods

Chemicals: Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) and Gibco (Grand Island, NY, USA), unless otherwise indicated.

Animals, housing, and maintenance: The animals used in this study were maintained in accordance with the guidelines of the committee on laboratory animals of Razi Institute. The mice were fed ad libitum with commercial autoclaved food and water. The animals were housed in 14:10 hour's light/dark cycle at 21±0.5°C and humidity 50 - 60%.

Oocyte collection, in vitro maturation, fertilization, and culture: Immature mouse oocytes were collected from 5 to 6-week-old NMRI mice. Mice were stimulated by an i.p. injection of 5 IU pregnant mare serum gonadotropin (PMSG, Folligon; Intervet, Castle Hill, NSW, Australia). The animals were killed 45 hours later by cervical dislocation and immature oocytes were released by puncturing the follicles under a stereomicroscope. After 3 to 4 washes in human tubal fluid (HTF) medium, immature oocytes surrounded by compact cumulus cells were selected; rinsed once in maturation medium (Minimum Essential Medium alpha with Earle salts (MEM-alpha, Gibco) plus 10% (v/v) heat inactivated FCS and penicillin G (50 IU/mL) and streptomycin sulphate (50 µg/mL) and finally placed in 4-well culture dishes (Nunclon, Nalgene, Nunc International, Roskilde, Denmark) at 37°C in 5% CO2 in air and maximum humidity for 17 hours. After IVM, matured oocytes were transferred into the droplets of HTF as fertilization medium. For fertilization, all oocytes were denuded, and cumulus free oocytes were transferred into the medium. Sperm samples were collected from the epididymis and vas deferens of 16-month-old B6D2F1 male mice and allowed to capacitate in the fertilization medium for 30 to 40 min prior oocyte-sperm co-culture. The final concentration of spermatozoa used during IVF was 1×10^6 motile spermatozoa/mL. Gametes were co-incubated in HTF medium, for 4 to 6 h at 37°C in 5% CO2 in air and maximum humidity. After gametes co-incubation, zygotes were washed and cultured in CZB culture medium supplemented with 1% (v/v) non-essential and 2% (v/v) essential amino acids at 37°C in 5% CO2 in air and maximum humidity. The medium was refreshed after 44 to 48 hours of culture.

Embryo evaluation: About 6h after the beginning of fertilization, oocytes were observed under an invert microscope and only 2PN zygotes were counted. During in vitro culture, embryo development was evaluated every 24h and 2-cell, 4-cell, 8-cell, morula, and blactocyst embryos were recorded. Blastocyst development was monitored on day 5, and blastocysts morphology was classified as

shown in Table 1 (Van soom and Boerjan).

Experimental design: Experiment 1. The effect of all-trans retinol during IVF on mouse embryo development and morphology

This experiment was conducted using 4 groups. The experiment was performed to examine the effect of all-trans retinol during IVF on mouse embryo development and morphology. Matured oocytes and sperm were co-cultured in fertilization medium containing different concentrations of all-trans retinol (0, 1, 5, and 10 μM). After fertilization, zygotes in each group were cultured in CZB culture medium for 5 days. In this experiment, percentage of 2-cell, 4-cell, 8-cell, morula, and blactocyst embryos and blastocyst morphology classification were recorded.

Experiment 2. The effect of all-trans retinol during IVC on mouse embryo development and morphology

This experiment was conducted on 4 groups. The experiment was performed to examine the effect of all-trans retinol during IVC on mouse embryo development and morphology. In this experiment, zygotes were cultured in CZB culture medium containing different concentrations of all-trans retinol (0, 1, 5, and 10 μM) for 5 days. In this experiment, percentage of 2-cell, 4-cell, 8-cell, morula, and blactocyst embryos and blastocyst morphology classification were recorded.

Statistical analysis: Data were analyzed by proc GLM of SAS and Duncan's test for mean differences ($p<0.05$). Percentage data were transformed using the arcsine transformation before analysis.

Results

Experiment 1. The effect of all-trans retinol during IVF on mouse embryo development and morphology

In this experiment, 5 μM all-trans retinol in the IVF medium significantly increased ($p<0.05$) mouse embryo development from 2-cell to blastocyst stage (Table 2) and grade one embryo compared to those in the presence of 1 μM all-trans retinol and control groups (Table 3).

Experiment 2. The effect of all-trans retinol during IVC on mouse embryo development and morphology

In this experiment, mouse embryo development was not altered by different concentrations of all-trans retinol (Table 2); however, the percentage of grade one embryos was higher in the presence of 10 μM all-trans retinol compared to that of the control group (Table 3).

Discussion

In this study, all-trans retinol was used in fertilization and embryo culture media to investigate its effect on embryo development and morphology. Administration of all-trans retinol during the IVF period showed a dose-dependent effect. The presence of 10 μM all-trans retinol had no effect on blastocyst formation rate, whereas using 1 and 5 μM all-trans retinol improved blastocyst formation compared to that in control group. Similarly, exposure of bovine oocytes to the low concentrations of all-trans retinol improved blastocyst formation rate; however, high concentrations had deleterious effects (Gomez et al., 2003). This improving effect could be due to the anti-oxidation effects of all-trans retinol during fertilization. Mammalian cells, including oocyte and embryo, have several mechanisms for protecting against ROS deleterious effects. Vitamins A, C, and E, pyruvate, glutathione, hypotaurine, taurine, and cysteamine are functional antioxidants in oocyte and embryo (Guerin et al., 2001). The addition of retinol to embryo culture medium can improve development to the blastocyst stage when cultured in a high pressure of O2 (20% O2), but not in a low pressure of O2 (7% O2). Previous in vitro experiments demonstrated a positive effect of retinoid during maturation with high pressure of O2 (Gomez et al., 2003; Hidalgo et al., 2003). It seems that retinoid may protect embryos from the oxidative stress, which has been identified as a reason of embryonic impairment (Guerin et al., 2003). The treatment of cumulus-oocyte complexes with all-trans retinol during meiotic arrest was observed to improve cortical granule migration, increase blastocyst formation, and total cell number (Duque et al., 2002). It has been suggested that retinoid may improve mRNA quality, because it has been observed that all-trans retinol increased poly-(A) mRNA content in meiotically arrested oocytes (Gomez et al., 2003). The addition of 1 and 5 μM all-trans retinol increased embryo

Table 1. Morphological evaluation in mouse embryo.

Embryo grade	Specification
Grade 1. Excellent embryo	embryos with monotonous, spherical, and moderate transparent blastomers
Grade 2. Good embryo	embryos with monotonous, spherical blastomers, and less than 10 % fragmentation
Grade 3. Moderate embryo	embryos with abnormal blastomers with no fragmentation
Grade 4. Weak embryo	embryos with normal or abnormal blastomers and more than 50 % fragmentation
Grade 5. Non-utilizable embryo	completely fragmentized embryos

Table 2. Mean (±SD) of pre-implantation development of mouse embryos in different groups. $^{(a,b,c)}$ Means with different superscript letters are different at $p<0.05$ in each column for each experiment.

Experiment	All-trans retinol concentration (µM)	Number of oocyte	2PN oocyte	2-cell embryo	4-cell embryo	8-cell embryo	Morula embryo	Blastocyst embryo
1	0	477	75.18 ± 1.82^{a}	69.56 ± 1.41^{b}	63.92 ± 1.43^{b}	58.24 ± 1.15^{b}	55.80 ± 1.67b	52.07 ± 1.51c
	1	485	75.68 ± 2.55^{a}	69.33 ± 2.16^{b}	64.46 ± 2.75^{b}	60.10 ± 2.50^{b}	57.96 ± 2.88b	59.85 ± 1.54b
	5	495	79.70 ± 1.13^{a}	78.58 ± 1.34^{a}	77.00 ± 1.21^{a}	74.89 ± 1.35^{a}	73.47 ± 1.83^{a}	68.50 ± 0.21^{a}
	10	468	74.08 ± 1.27^{a}	65.70 ± 1.77^{b}	63.46 ± 1.69^{b}	58.71 ± 1.65^{b}	57.52 ± 1.21^{b}	53.90 ± 1.01^{bc}
2	0	495	75.36 ± 1.91^{a}	66.18 ± 0.69^{a}	63.46 ± 0.80^{a}	60.61 ± 1.56^{a}	58.25 ± 1.70^{a}	52.50 ± 1.49^{a}
	1	470	76.46 ± 2.02^{a}	71.08 ± 2.25^{a}	67.96 ± 3.02^{a}	61.62 ± 2.68^{a}	57.79 ± 2.28^{a}	55.20 ± 3.07^{a}
	5	472	72.70 ± 3.20^{a}	68.76 ± 3.52^{a}	66.26 ± 1.52^{a}	62.43 ± 1.21^{a}	59.82 ± 1.47^{a}	58.41 ± 1.67^{a}
	10	488	76.47 ± 3.39^{a}	66.83 ± 1.67^{a}	64.60 ± 0.75^{a}	60.12 ± 1.70^{a}	57.81 ± 2.36^{a}	54.03 ± 2.26^{a}

Table 3. Mean (±SD) of different grades of mouse blastocyst embryos according to the morphological classification in different groups. (a,b,c) Means with different superscript letters are different at $p<0.05$ in each column for each experiment.

Experiment	All-trans retinol concentration (µM)	Number of blastocyst embryos	Grade one	Grade two	Grade three	Grade four	Grade five
1	0	248	35 ± 0.02^{bc}	25 ± 0.02^{a}	17 ± 0.02^{a}	14 ± 0.03^{a}	10 ± 0.01^{a}
	1	290	46 ± 0.02^{b}	19 ± 0.03^{a}	18 ± 0.04^{a}	11 ± 0.03^{a}	8 ± 0.02^{ab}
	5	339	55 ± 0.04^{a}	28 ± 0.05^{a}	9 ± 0.01^{b}	8 ± 0.02^{a}	4 ± 0.01^{b}
	10	253	48 ± 0.02^{ab}	18 ± 0.02^{a}	12 ± 0.02^{ab}	15 ± 0.04^{a}	9 ± 0.01^{a}
2	0	260	39 ± 0.02^{b}	24 ± 0.02^{a}	16 ± 0.01^{a}	13 ± 0.01^{a}	9 ± 0.02^{a}
	1	259	42 ± 0.03^{ab}	24 ± 0.01^{a}	16 ± 0.01^{a}	10 ± 0.01^{a}	9 ± 0.03^{a}
	5	275	46 ± 0.01^{ab}	26 ± 0.03^{a}	13 ± 0.01^{a}	8 ± 0.01^{a}	8 ± 0.01^{a}
	10	263	49 ± 0.03^{a}	20 ± 0.02^{a}	16 ± 0.01^{a}	10 ± 0.01^{a}	$7 \pm .01^{a}$

development from 2-cell embryo to the blastocyst stage. One of the probable reasons for this improvement may be the beneficial effect of all-trans retinol on cumulus cells. The increase of grade one blastocyst by 5 µM all-trans retinol during IVF period is probably due to a decrease of oxidative stress and apoptosis in zygotes and embryos. However, the mechanism by which all-trans retinol affects oocyte fertilization and embryonic development is not known. Retinoids participate in the biological antioxidant process and have been implicated as important regulators of redox signaling pathways. Carotenoids and retinol can scavenge single oxygen molecules and interact with the other antioxidant compounds (Olson, 1993). Retinoic acid has been shown to protect against oxidative stress-induced apoptosis by inhibition of the c-jun N-terminal kinase activator protein 1 pathway in some cells (Konta et al., 2001; Moreno-Manzano et al., 1999). These data provide documents that in many cell systems retinoid can improve antioxidant protection mechanisms. On the other hand, different concentrations of all-trans retinol in embryo culture medium failed to increase mouse embryo development; however, it could

improve grade one blastocyst compared to that of the control group. It seems that modification in embryo culture medium and/or condition has more effect on embryo morphology than on blastocyst formation (Zhandi et al., 2010).

In conclusion, the results of this study showed that administration of all-trans retinol during IVF can increase embryo development to blastocyst stage and improve blastocyst morphology, but its administration during IVC can only improve blastocyst morphology.

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اثر آل-ترانس رتینول بر تکامل برون تنی رویان موش آزمایشگاهی

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چکیده

زمینه مطالعه: آل-ترانس رتینول یک‌آنتی اکسیدان زیستی است که می‌تواند رادیکال‌های آزاد اکسیژن را در محیط‌های کشت سلولی جمع‌آوری کند. **هدف:** در این مطالعه، اثرافزودن آل-ترانس رتینول در محیط کشت لقاح ورشدرویان برتکامل رویان موش آزمایشگاهی در طی دوآزمایش ارزیابی شد. **روش کار:** درآزمایش نخست، اووسیت هایی که پیشتردر محیط کشت بالغ شده بودند، درکناراسپرم در درون محیط لقاح آزمایشگاهی حاوی غلظت‌های ۰µL، ۱، ۵ و۱۰آل-ترانس رتینول قرار داده شدند. پس ازلقاح، زیگوت‌های حاصله بطور جداگانه درمحیط CZBبه مدت ۵ روز تا مرحله بلاستوسیست کشت داده شدند. درآزمایش دوم، زیگوت‌های حاصل در شرایط برون تنی، در محیط کشت CZBکه حاوی غلظت‌های ۰µL، ۱، ۵ و۱۰آل-ترانس رتینول بود، به مدت ۵ روز تا مرحله بلاستوسیست قرار داده شدند. **نتایج:** درآزمایش نخست، نرخ تشکیل بلاستوسیست به طور معنی‌داری در گروه ۵µL بیشتر از بقیه گروه‌ها بود. همچنین، درصد رویان‌های باکیفیت عالی (درجه یک) در این گروه به طور معنی‌داری بیشتر از گروه‌های ۰µLو۱ بود. درآزمایش دوم، غلظت‌های مختلف‌آل-ترانس رتینول تغییری در نرخ تشکیل بلاستوسیست ایجاد نکرد، اما درصد رویان‌های درجه یک در گروه ۱۰µL بیشتر از شاهد بود. **نتیجه‌گیری نهایی:** نتایج این آزمایش نشان می‌دهد که افزودن ۵µL آل-ترانس رتینول در محیط لقاح‌آزمایشگاهی می‌تواند نرخ تکامل و خصوصیات ریخت شناختی رویان‌ها را بهبود بخشد. از طرفی افزودن این ماده به محیط کشت رویان می‌تواند بدون تغییر در نرخ تکامل رویان موش آزمایشگاهی، سبب بهبود ویژگی‌های ریخت‌شناختی آن شود.

واژه‌های کلیدی: تکامل، لقاح، موش، اووسیت، رتینول

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