Effects of all-trans retinoic acid on the *in vitro* maturation of camel (*Camelus dromedarius*) cumulus-oocyte complexes

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**Abstract.** All-trans retinoic acid (RA) is a metabolite of vitamin A and has pleiotropic actions on many different biological processes, including cell growth and differentiation, and is involved in different aspects of fertility and developmental biology. In the current study, we investigated the effects of RA on camel (*Camelus dromedarius*) cumulus-oocyte complex *in vitro* maturation (IVM). IVM medium was supplemented with 0, 10, 20, and 40 µM RA. Application of 20 µM RA significantly reduced the proportion of degenerated oocytes and significantly improved oocyte meiosis and first polar body extrusion compared to the control and other experimental groups. Retinoic acid significantly reduced the mRNA transcript levels of apoptosis-related genes, including BAX/BCL2, BAX, and P53, and reduced the BAX/BCL2 ratio. In addition, RA significantly reduced the expression of the Transforming growth factor beta (TGFβ) pathway-related transcripts associated with the actin cytoskeleton, ACTA2 and TGLN; however, RA increased TGFβ expression in cumulus cells. The small molecule SB-431542 inhibits the TGFβ pathway by inhibiting the activity of activin receptor-like kinases (ALK-4, ALK-5, and ALK-7); however, combined supplementation with RA during IVM compensated for the inhibitory effect of SB-431542 on cumulus expansion, oocyte meiosis I, and first polar body extrusion in activated oocytes. The current study shows the beneficial effects of RA on camel oocyte IVM and provides a model to study the multifunctional mechanisms involved in cumulus expansion and oocyte meiosis, particularly those involved in the TGFβ pathway.

**Key words:** All-trans retinoic acid, Apoptosis, Oocytes, SB-431542, Transforming growth factor beta (TGFβ)

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Camels (*Camelus dromedarius*) are xeric animals and, in extremely arid conditions, are better sources of meat and milk than are cattle. However, the low reproductive performance of camels is considered an important factor affecting camel populations. Employing *in vitro* embryo production (IVP) technologies is an efficient means of propagating genetically superior camels; however, compared to other livestock species, camel oocyte *in vitro* maturation (IVM) has been reported to be inefficient [1–4]. One of the main contributing factors is the limited availability of oocytes because the majority of the slaughtered animals are either old, culled for infertility, or very young and have not attained maturity; in addition, there is no standardized protocol for IVP in camels [3, 5]. Therefore, refinement of the camel IVP system and oocyte maturation is necessary, especially when the availability of oocytes is limited [6, 7].

Retinoic acid (RA) is a metabolite of vitamin A (retinol) that mediates the functions of vitamin A, such as cell growth, development, and differentiation, and has been implicated in reproductive processes including folliculogenesis and embryonic survival [8]. In the last two decades, several reports have revealed the crucial role of the two major retinol metabolites, all-trans and 9-cis retinoic acid, on oocyte maturation and its developmental competence in different mammalian species, both *in vivo* and *in vitro* [9–21]. However, to the best of our knowledge, there are no reports regarding the effects of RA on camel oocytes. Retinoic acid has multifunctional roles during *in vitro* oocyte maturation; it may promote cytoplasmic maturation through its modulatory effects on the expression of genes encoding gonadotrophin receptors, midkine, cyclooxygenase 2, and nitric oxide synthase in cumulus-granulosa cells [11, 13]. Another potential role for RA is in reducing apoptosis in cumulus-oocyte complexes [13, 20]; in addition, RA may also promote cumulus expansion [14, 16]. Despite the presence of RA receptors in cumulus cells [22], RA may act directly to improve oocyte maturation in the absence of cumulus cells [23], or act as an antioxidant to reduce the levels of reactive oxygen species and oxidative stress during oocyte maturation [11, 24, 25].

Transforming growth factor beta (TGFβ) superfamily members are involved in oocyte maturation either directly [26] or indirectly...
through cumulus expansion and differentiation [27], or through a bidirectional interplay between oocytes and surrounding cumulus cells [28, 29]. Studies have shown the effects of RA on TGFβ pathways in cells other than cumulus-oocyte complexes, such as in osteoblasts [30], smooth muscle cells [31, 32], and mucosal cells [33]. Therefore, in the present study, we investigated the effects of all-trans RA on camel cumulus-oocyte complex (COC) IVM by examining cumulus expansion, nuclear maturation, and mRNA transcript levels of apoptosis, gap junction, and TGFβ pathway-related genes involved in cell cytoskeleton integrity, in both oocytes and cumulus cells.

Materials and Methods

Chemicals
Chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated.

In vitro maturation
Camel ovaries (n = 320) were collected from a local slaughterhouse from October 2017 to March 2018. The ovaries were transported in normal saline solution at 30–33°C to the laboratory within 3–6 h. Antral follicle (2–8 mm in diameter) contents were aspirated with 18-gauge needles connected to 10-ml disposable syringes. Cumulus-oocyte complexes showing an even granulated cytoplasm and enclosed in compact cumulus cells were selected (a total of 1550 eligible COCs were used in the study). They were then washed three times with tissue culture medium-199 (TCM-199) supplemented with 2 mM NaHCO3, 25 mM HEPES, 0.1% bovine serum albumin (BSA), and 5 µg/ml gentamycin sulfate. The COCs were then divided into four groups (20–25 oocytes each) according to the experimental design and cultured in 4-well dishes in 500 µl maturation medium at 38.5°C in a humidified atmosphere with 5% CO2 for 28 h. The IVM medium comprised of TCM-199 supplemented with 10 µg/ml follicle-stimulating hormone, 10 µg/ml luteinizing hormone, 1 µg/ml 17β-estradiol, 20 ng/ml epidermal growth factor, 1 µL/ml insulin-transferrin-selenium, 0.3 µM cysteamine, 0.15 mg/ml L-glutamine, 10% fetal bovine serum (FBS), and 5 µg/ml gentamycin sulfate [6].

RA and SB-431542 supplementation
A 50 mM stock of all-trans RA (R2625, Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO) was stored at −20°C until further use. For the experiments, the all-trans RA stock was diluted in IVM medium to 0, 10, 20, and 40 µM. The solvent with DMSO at a final concentration of < 0.02% was used as the vehicle control. SB-431542 at 20 µM [34] was supplemented either individually or combined with RA as explained in the experimental design.

Evaluation of cumulus cell expansion and oocyte nuclear maturation
After IVM, the COCs were categorized according to the degree of cumulus expansion as follows: grade 0, no expansion was observed; grade 1, partially expanded cumulus; and grade 2, completely expanded cumulus with clear intercellular spaces around the oocytes as shown in Fig. 1 [7]. For oocyte degeneration and oocyte nuclear assessment (the meiotic transition from metaphase I, anaphase I, telophase I, and metaphase II; Fig. 2), cumulus cells were separated from oocytes by pipetting in TCM-199 supplemented with 1 mg/ml hyaluronidase and washed three times in TCM-199 supplemented with 10% FBS. The oocytes were then stained with 5 µg/ml bisbenzimide (Hoechst 33342) for 5 min to visualize the nuclei and polar body extrusions using an inverted microscope equipped with epifluorescence [6].

Parthenogenesis of mature oocytes and embryo culture
Oocytes were stripped from surrounding cumulus cells by pipetting in TCM-199 supplemented with 1 mg/ml hyaluronidase and washed three times in TCM-199 supplemented with 10% FBS. Oocytes extruding the first polar body were selected and activated in TCM-199 through cumulus expansion and differentiation [27], or through a bidirectional interplay between oocytes and surrounding cumulus cells [28, 29]. Studies have shown the effects of RA on TGFβ pathways in cells other than cumulus-oocyte complexes, such as in osteoblasts [30], smooth muscle cells [31, 32], and mucosal cells [33]. Therefore, in the present study, we investigated the effects of all-trans RA on camel cumulus-oocyte complex (COC) IVM by examining cumulus expansion, nuclear maturation, and mRNA transcript levels of apoptosis, gap junction, and TGFβ pathway-related genes involved in cell cytoskeleton integrity, in both oocytes and cumulus cells.

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supplemented with 10% FBS and 5 µM ionomycin for 5 min [6, 7].

Activated oocytes (15 oocytes per treatment, five replicates) were washed three times in TCM-199 with 10% FBS, transferred to 50 µl microdrops of 4 mM 6-dimethylaminopurine (6-DMAP), overlaid with mineral oil, and cultured for 4 h in an atmosphere of 5% CO2, 5% O2, and 90% N2 at 38.5°C. Activated oocytes were then washed in the culture medium and distributed in freshly prepared KSOMaa culture medium (one oocyte/5 µl) covered with mineral oil, and cultured in an atmosphere of 5% CO2, 5% O2, and 90% N2 at 38.5°C. Cleavage and subsequent embryo development were evaluated on days 2 and 7 of IVC, respectively. The cleavage proportion was calculated as a percentage of the number of oocytes showing first polar body extrusion. Blastocyst development was calculated as the percentage of cleaved embryos.

Relative quantification of cumulus and oocyte mRNA transcript levels

Cumulus cells were stripped from the oocytes and washed with centrifugation at 3000 rpm for 2 min in phosphate-buffered saline (PBS). Oocytes (n = 20, three replicates) were washed in PBS. The experiment was repeated three times. Total RNA was extracted from cumulus cells and oocytes using an RNA extraction Kit (iNtRON Biotechnology, Daegu, South Korea) according to the manufacturer’s instructions. A NanoDrop™ 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA) was used to estimate RNA concentration and purity. Pulsed reverse transcription (RT) was performed on the extracted RNA, according to Saadedin et al. [35]: 80 cycles of 2 min at 16°C, 1 min at 37°C, 0.1 s at 50°C, followed by final inactivation at 85°C for 5 min. Individual RT reactions were performed using a SuperScript™ III First-Strand Synthesis System (Thermo Fischer) according to the manufacturer’s instructions, using 30 ng of RNA and random hexamers in a 20 µl reaction volume. Relative quantification of mRNA transcripts was performed using real-time PCR (ViiA 7, Applied Biosystems, Foster City, CA, USA). The reactions contained 100 ng cDNA, 1 µM forward primer, 1 µM reverse primer, and 1 × SYBR® Green Premix (Applied Biosystems). The reactions were run in triplicate and GAPDH was used as a reference gene. The fold change and relative quantities of apoptosis-related (P53, BAX, and BCL2), gap junction CX43, and TGFβ pathway-related (TGFB, TAGLN, and ACTA2) transcripts were calculated using the 2−ΔΔCt method [36]. Reactions without cDNA template or reverse transcriptase resulted in no amplification. Cumulus cells and oocytes of the control group were set as the arbitrary group to calculate the fold change in mRNA expression. Thermal cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec, and 72°C for 40 sec. PCR products were fractioned on 1.5% agarose gel using a 1 kb DNA ladder (iNtRON Biotechnology) as a reference and stained with RedSafe™ (iNtRON Biotechnology). Table 1 shows the primer sequences, annealing temperatures, and approximate product sizes of the amplified fragments of the candidate genes.

Experimental design

Experiment 1 was conducted to investigate four different concentrations of RA (0 [control], 10, 20, and 40 µM) on COC in vitro maturation, cumulus expansion, and oocyte developmental competence after parthenogenetic activation. For IVM, a total of 500 IVM oocytes were used, 25 oocytes per treatment with 5 replicates. For parthenogenetic activation, a total of 300 activated oocytes were used, 15 activated oocytes per treatment with 5 replicates. Moreover, the relative quantification of mRNA transcripts of apoptosis-related genes, the CX43 gene, and TGFβ-related cell integrity pathway genes was evaluated between control and 20 µM RA-supplemented oocytes (20 oocytes per group with 3 replicates).

Experiment 2 was conducted to investigate the ameliorative or compensatory effects of RA on TGFβ inhibitor (SB-431542)-treated COCs. A total of 500 COCs were distributed between four groups (25 COCs each group, 5 replicates): control, 20 µM SB-431542 (SB), 20 µM RA, and RA + SB. Cumulus expansion and nuclear status were assessed in each group after 28 h of IVM.
Statistical analysis

COCs were randomly assigned to the designed experimental groups, and the experiments were repeated at least five times. Oocyte maturation values, degree of cumulus expansion, and embryo development (cleavage and blastocyst) from each trial are presented as means ± SEM, and values were analyzed using one-way analysis of variance (ANOVA). Quantitative PCR values were also compared by ANOVA. The \( \text{BAX} / \text{BCL2} \) values were calculated for all replicates and are presented as the means ± SEM and compared using ANOVA. Differences were considered significant at \( P < 0.05 \).

Results

Effect of RA on COC in vitro maturation

Supplementation of RA at 20 µM elicited a significant increase in the proportion of oocytes showing an extruded first polar body (69.43 ± 4.6%) compared to the control and other experimental groups (Table 2). Moreover, supplementing RA at 20 µM significantly decreased the proportion of degenerated oocytes (19.01 ± 1.9%) compared to the control and other experimental groups (Table 2). No differences were found between the experimental groups in cumulus expansion, cleavage percentage, and blastocyst percentage.

Effects of RA on cumulus and oocyte mRNA transcript levels

Retinoic acid (20 µM) application significantly reduced the expression of \( \text{BAX}, \text{BCL2}, \) and \( \text{P53} \) in both cumulus cells and oocytes compared to the control group (Fig. 3). However, RA elicited no effect on the expression of \( \text{CX43} \) in cumulus cells and oocytes (Fig. 4). Moreover, RA application significantly reduced \( \text{ACTA2} \) and \( \text{TAGLN} \) expression in both cumulus cells and oocytes, whereas \( \text{TGFβ1} \) expression was significantly increased in cumulus cells with RA supplementation (Fig. 5).

Table 1. Primers used for quantitative PCR

| Gene   | Forward primer (5’→3’) | Reverse primer (5’→3’) | Product size (bp)* | Accession No.   |
|--------|------------------------|------------------------|--------------------|-----------------|
| BAX    | AGATCATGAAGACAGGGGCC  | GCGATCATCCTGGAACCTC    | 190                | XM_010996357.1  |
| BCL-2  | CAGGCTCAACGTTGCAATCAG | TAGGTTGGGCTGGCAATTA    | 151                | XM_010979933.1  |
| P53    | AGCTCTCTCCACCAACAAA  | GTGAGCTTTTCTTTCCCTTG  | 159                | XM_010996514.1  |
| ACTA2  | CAGGGCTGTTTCCCCATCTA | TTTTGGTGGTGGCTCAC    | 104                | XM_010996278    |
| TAGLN  | CTACACGCAATCTGCTATCC | CCAACAGGACAGTGGACCT   | 148                | XM_010981405    |
| TGFβ1  | TGGCTGTTTGGTGGTAC    | CCGGTCGTTAAGTCCTAC    | 135                | XM_010985237    |
| CX43   | TCAGGTTGACCTGCTCTCTC | TCTTCCCTTGACAGATG    | 149                | XM_010979171    |
| GAPDH  | TGCTGAGTACCTTTGGGAGT | TCACGCCCATACAAACATG   | 134                | XM_010990867    |

* The melting curve for each primer was evaluated by Viia™7 apparatus-associated software and the product size was confirmed by gel electrophoresis of PCR products on 1.5% agarose gels using a 1 kb DNA ladder as a reference.

Table 2. Effects of different concentrations of all-trans retinoic acid (RA) on camel oocyte in vitro maturation and subsequent parthenogenetic embryo development

| No. of IVM oocytes | Control | RA (10 µM) | RA (20 µM) | RA (40 µM) |
|--------------------|---------|------------|------------|------------|
| First polar body extrusion (%) | 65 (52.95 ± 4.2)² | 65 (53.01 ± 3.3)² | 86 (69.43 ± 4.6)³ | 64 (52.72 ± 2.01)³ |
| No polar body (%) | 5 (2.9 ± 1.0) | 5 (3.7 ± 0.8) | 6 (4.12 ± 1.1) | 6 (4.03 ± 1.3) |
| Metaphase I | 5 (2.5 ± 0.9) | 5 (3.63 ± 0.9) | 5 (3.54 ± 1.2) | 6 (4.03 ± 1.3) |
| Anaphase I | 6 (3.19 ± 1.1) | 5 (3.1 ± 1.0) | 5 (3.9 ± 0.9) | 5 (3.8 ± 0.9) |
| Degenerated oocytes (%) | 44 (38.46 ± 2.1)⁴ | 45 (36.56 ± 2.4)⁴ | 23 (19.01 ± 1.9)⁵ | 45 (35.42 ± 1.5)⁴ |
| No. of activated oocytes | 75 | 75 | 75 | 75 |
| Cleavage (%) | 45 (60.52 ± 3.2) | 44 (58.43 ± 3.1) | 45 (61.05 ± 2.1) | 44 (59.8 ± 3.2) |
| Blastocyst (%) | 5 (12.33 ± 2.7) | 6 (13.21 ± 2.1) | 6 (13.5 ± 1.3) | 5 (12.16 ± 2.0) |

* No polar body; morphologically normal with no completion of first meiosis and still in metaphase I, anaphase I, or in pro-telophase I as illustrated in Fig. 2. ² Cleavage ratio was calculated as the percentage of > 2-cell stage resulting from parthenogenetically activated first polar body oocytes. ³ Blastocyst ratio was calculated as the percentage of cleaved embryos.

Discussions

Several studies have revealed the crucial role of all-trans retinoic
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However, to the best of our knowledge, there are no studies on the effects of RA on camel oocytes. Retinoic acid supplementation significantly decreased the proportion of degenerated oocytes (Table 2), which might be attributed to the decreased expression of BAX, BAX/BCL2, and P53 observed in the current study (Fig. 3). This result coincides with the decreasing effects of RA on apoptosis-related genes in canine and caprine oocytes [13, 20].

However, RA supplementation had no effect on the expression of CX43, which encodes a gap junction protein, in both oocytes and cumulus cells (Fig. 4). This result contradicts those obtained by Best et al. 2015 and Pauli et al. 2010 [37, 38] who showed that retinoids upregulate CX43 expression. In these two studies, the authors investigated the effects of retinoids on primary cumulus granulosa cells without oocytes in the culture conditions. This major difference in experimental design between the studies may indicate a bidirectional paracrine effect between oocytes and cumulus cells to stabilize CX43 expression mediated by RA.

Fig. 3. Effect of all-trans retinoic acid (RA) on the relative quantification (RQ) of BAX, BCL2, and P53 mRNA transcripts in camel cumulus cells and oocytes after IVM. The fold change (RQ) in mRNA transcripts was normalized to GAPDH levels for each treatment. Relative mRNA expression levels in the control group were arbitrarily set as 1. Fold change of the BAX/BCL2 ratio was calculated for cumulus cells and oocytes and is presented as the mean ± SEM. Asterisks (*) indicate significant differences (P < 0.05) compared to control.

Fig. 4. Effects of all-trans retinoic acid (RA) on the relative quantification (RQ) of CX43 mRNA transcripts in camel cumulus cells (Cum) and oocytes (OO) after IVM. The fold change (RQ) in CX43 transcript levels was normalized to GAPDH levels in cells for each treatment. Data are presented as means ± SEM. Relative mRNA expression levels in the control group were arbitrarily set as 1.

Fig. 5. Effects of all-trans retinoic acid (RA) on the relative quantification (RQ) of ACTA2, TAGLN, and TGFβ mRNA transcripts in camel cumulus cells and oocytes after IVM. The fold change (RQ) in mRNA transcript levels was normalized to GAPDH levels in cells for each treatment. The mRNA expression levels in the control group were arbitrarily set as 1. Data are presented as means ± SEM. Asterisks (*) indicate significant differences (P < 0.05) between transcripts.
Additionally, RA supplementation increased TGFβ expression in cumulus cells (Fig. 5). The increase in TGFβ expression enhances oocyte development, steroidogenesis, and aromatization by cumulus granulosa cells, and upregulate essential transcripts required for oocyte meiosis [27, 39–41]. Paradoxically, RA significantly reduced granulosa cells, and upregulate essential transcripts required for cell differentiation [31, 32], and bronchial epithelium [44]; however, RA inhibits TGFβ signaling and restricts the expression of smooth muscle genes like ACTA2 and TGLN in distal lungs to prevent ectopic and excessive cell differentiation [31, 32]. TGLN and ACTA2 are TGFβ-inducible genes and are required for actin cytoskeleton organization and cellular differentiation [45]; however, their function in oocytes, cumulus cells, and ovarian cells is unknown and requires further investigation [42].

To investigate the effects of RA on the TGFβ pathway, we used SB-431542 to specifically inhibit the activin receptor-like kinase (ALK) 4/5/7, that can be other than activin receptor-like kinases (ALK) 4/5/7, that can be inhibited by research group RG-1438-066. The results of the aforementioned studies and show that SB-431542 application induced complete arrest of cumulus expansion and meiosis I (Table 3; Fig. 2). Interestingly, RA mitigated the inhibitory effect of SB-431542, improving cumulus expansion and activating the oocytes to complete meiosis I and first polar body extrusion (Table 3). This result indicates the importance of alternative pathways, other than activin receptor-like kinases (ALK) 4/5/7, that can be stimulated by RA in cumulus-oocyte bidirectional communication.

The current study showed the beneficial effects of all-trans RA on camel oocyte in vitro maturation in terms of a reduction in degenerated oocytes, an increase in first polar body extrusion, and a reduction in the expression of apoptosis-related transcripts. Moreover, RA overcame the SB-431542-mediated inhibition of ALK 4/5/7 and improved oocyte meiosis and cumulus expansion, indicating a molecular compensatory action for RA.

### Table 3. Effects of combined supplementation of all-trans retinoic acid (RA) with the TGFβ pathway inhibitor SB-431542 (SB) on camel oocyte in vitro maturation

|                      | Control  | SB          | RA       | RA + SB    |
|----------------------|----------|-------------|----------|------------|
| No. of oocytes       | 125      | 125         | 125      | 125        |
| First polar body (%) | 66 (53.6 ± 3.1) b | 14 (11.2 ± 2.5) c | 90 (71.9 ± 4.1) a | 66 (52.9 ± 3.4) b |
| No polar body (%)    | 5 (3.8 ± 0.8) b | 50 (40.08 ± 1.1) a | 4 (2.9 ± 0.6) b | 5 (4.2 ± 1.0) b |
| Metaphase I          | 5 (3.1 ± 0.9) b | 13 (10.0 ± 1.9) a | 4 (3.1 ± 0.7) b | 5 (3.8 ± 0.9) b |
| Anaphase I           | 5 (3.29 ± 1.1) b | 4 (3.0 ± 1.0) a | 4 (2.36 ± 0.9) b | 4 (2.32 ± 0.7) a |
| Telophase I          | 44 (36.21 ± 1.7) a | 44 (35.72 ± 0.8) a | 23 (19.74 ± 1.0) a | 45 (36.78 ± 1.1) a |
| Cumulus expansion degree b | 2.0 ± 0.0 a | 0.0 ± 0.0 a | 2.0 ± 0.0 a | 1.0 ± 0.0 b |

* No polar body; morphologically normal with no completion of first meiosis and still in metaphase I or anaphase I. The nuclear status and meiosis steps are shown in Fig. 2. The degree of cumulus expansion was calculated by observation of the five IVM replicates; representative images are shown in Fig. 1.

### Conflict of interest: The authors declare that they have no conflicts of interest.

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