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Interleukin-7 improves *in vitro* maturation of ovine cumulus-oocyte complexes in a dose dependent manner

Pradeep Krishna Javvaji[1], Arindam Dhali*, Joseph Rabinson Francis[1], Atul P. Kolte, Anjumoni Mech, L. Sathish[2], Sudhir C. Roy

ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru 560 030, India

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ABSTRACT

Interleukin-7 (IL-7) mediated signals are linked to development, proliferation, survival and differentiation of cells. Recent evidences indicate its role in oocyte maturation process as well. Nevertheless, the underlying mechanisms of IL-7 involvement in oocyte maturation are not well characterized. In addition, currently no information is available on the effect of exogenous IL-7 on oocyte maturation in ovine or any other species. In this study, the effect of IL-7 supplementation during *in vitro* maturation (IVM) on the maturation rate, production of reactive oxygen species (ROS) and gene expression of ovine cumulus-oocyte complexes (COC) was assessed. IL-7 (0.5, 1, 2, 5 and 10 ng/ml) was supplemented in IVM medium at the beginning (0 h) and maturation rate of COC was assessed at the completion of IVM (24 h). The maturation rate (%) was found significantly (P = 0.000) greater with the 1 ng/ml of IL-7 supplementation (69.5) than control (60.0). In contrast, the maturation rate was reduced significantly (P = 0.000) with the 2 (47.1), 5 (39.2) and 10 ng/ml (39.1) of IL-7 as compared to the control. The level of intracellular ROS in the matured COC was found considerably higher with the 5 ng/ml of IL-7 followed by 1 ng/ml of IL-7 and control. It was evident that in the presence of superoxide dismutase-inhibitor, 1 ng/ml of IL-7 did not stimulate oocyte maturation. In contrast, oocyte maturation was improved with 5 ng/ml of IL-7 supplementation in the presence of NADPH-oxidase-inhibitor. IL-7 supplementation influenced gene expression in COC in a dose and time dependant manner. The expression of genes related to ROS production and apoptosis were upregulated and the genes associated with antioxidant mechanisms were downregulated noticeably with the supplementation of 5 ng/ml of IL-7. In conclusion, IL-7 at low concentration was beneficial for oocyte maturation, which was likely mediated through the favourable level of intracellular ROS and antioxidant mechanisms. In contrast, the detrimental effects of greater IL-7 concentrations on oocyte maturation were possibly arbitrated through the ROS-mediated oxidative stress, compromised antioxidant mechanism and stimulated apoptotic signalling.

1. Introduction

The success of *in vitro* embryo production depends on the proper *in vitro* maturation (IVM) of an oocyte that determines its competence to develop into an embryo following *in vitro* fertilization (IVF) and culture (IVC). Enrichment of basic IVM condition can significantly improve the competence of oocytes and their development into morula and blastocyst stages following IVF and IVC [1]. Over the past decades, beneficial effects of supplementing various growth factors, hormones and other molecules in IVM medium on oocyte maturation have been demonstrated [2,3].

Interleukin-7 (IL-7) is a pleiotropic cytokine that stimulates the development, proliferation, survival and differentiation of lymphocytes [4]. IL-7 acts by binding to IL-7 receptor complex, a heterodimer consisting of IL-7 receptor alpha (IL7RA) and gamma (γc) chains [4]. The IL7RA is specific for IL-7, but the γc chain is shared by the receptors of other interleukins [4]. Binding of IL-7 to its receptor induces multiple signalling pathways that are involved in cell proliferation and survival [5]. It is suggested that exogenous IL-7 may be beneficial for preventing apoptosis of T cells, but at high concentration, IL-7 may trigger the process in macaques [6].

Previously it has been shown that IL-7-induced generation of

* Corresponding author at: OMICS Laboratory, ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Hosur Road, Bengaluru 560 030, India.
E-mail address: dhali72@gmail.com (A. Dhali).
[1] Center for Post Graduate Studies, Jain University, Bengaluru-560 001, India.
[2] Central Sericultural Research and Training Institute, Mahadevapura, Mysuru-570 008, India.

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reactive oxygen species (ROS) promotes survival of human T-cell acute lymphoblastic leukemia cells [7]. ROS are essential for cellular functioning and they are controlled by antioxidants. ROS are known to be involved in stimulating PI3K-AKT signalling, which is important for cell survival, proliferation and metabolism [7]. Nevertheless, high ROS production can trigger apoptosis through the oxidative damage to cells in human [8]. The extent of ROS-mediated damage to cell depends on the balance between their production and removal rates. The involvement of ROS in ovulation, fertilization and conception has been suggested in rat, mouse, porcine and human [9]. An adequate ROS balance is important for oocyte quality. ROS is beneficial for meiotic resumption of oocytes at low concentration, but high ROS level is associated with meiotic cell cycle arrest and apoptosis in rat and human [9].

The possible involvement of IL-7 in the process of oocyte maturation is evident. The level of polysome bound *IL-7* mRNA increases significantly in human oocytes at M-II stage [10]. An association between the expression of *IL-7* in cumulus-oocyte complexes (COC) and oocyte developmental competence has been demonstrated in mouse [11]. It is suggested that oocyte-derived IL-7 can act on neighbouring granulosa cells as a survival factor and promote the nuclear maturation of preovulatory oocytes in rat [12]. Despite the above-mentioned knowledge, the underlying mechanisms of IL-7 involvement in oocyte maturation are not well characterized. In addition, currently no information is available on the effect of exogenous IL-7 on oocyte maturation in ovine or any other species.

The present study was designed to assess the effect of IL-7 supplementation during IVM on the maturation rate, ROS production and expression of the genes associated with mitochondrial DNA (mtDNA) synthesis, ROS production, antioxidant enzymes, apoptosis and cell proliferation in ovine COC.

2. Materials and methods

All the chemicals used in the experiments were procured from Sigma-Aldrich Co., St. Louis, MO, USA unless otherwise mentioned.

2.1. IVM of COC with IL-7

The effect of IL-7 supplementation on maturation of ovine COC was assessed in this experiment. COC were cultured *in vitro* for 24 h in IVM medium without (control) or with 0.5, 1, 2, 5 and 10 ng/ml of IL-7 supplementation (treatments). Oocyte maturation rate was assessed at the end of IVM and compared among the control and treatment groups. The experiment was conducted in triplicate. The total number of COC cultured and evaluated for maturation were 131, 126, 127, 122, 124 and 128, respectively in the control and 0.5, 1, 2, 5, and 10 ng/ml of IL-7 supplemented groups.

2.1.1. Collection of COC and IVM

Sheep ovaries were collected from a local abattoir and transported to the laboratory in a thermos containing warm (~37 °C) normal saline supplemented with strepto-penicillin (1.6 g/l; Cadila Healthcare Ltd., Vadodara, India) within 3–4 h of slaughter. Ovaries were washed thoroughly with strepto-penicillin supplemented warm normal saline and excess tissues were removed. COC were aspirated from the 2–6 mm follicles using a 20 G needle attached with a 2 ml syringe containing 500 µl of aspiration medium that was composed of HEPES-buffered M199 (Life Technologies Corporation, NY, USA) supplemented with heparin (50 IU/ml), gentamicin (50 µg/ml; Life Technologies Corporation, NY, USA) and fatty acid free BSA Fraction V (4 mg/ml). COC with more than five layers of compact cumulus cells and homogeneous cytoplasm were collected for the experiments.

The COC were washed in aspiration medium and then in B199 medium that was composed of bicarbonate-buffered M199 (Life Technologies Corporation, NY, USA) supplemented sodium pyruvate (0.2 mM), gentamicin (50 µg/ml), cysteamine (0.1 mM) and FBS (10%; Life Technologies Corporation, NY, USA). Ten COC in 10 µl of B199 medium were transferred into 40 µl drops of IVM medium (B199 supplemented with ovine-FSH, human-LH and 17b-estradiol) that were overlaid with mineral oil and pre-incubated for at least 2 h at 38.5 °C in a CO2 incubator (5% CO2 in a humidified environment). The final concentrations of FSH, LH, estradiol and FBS in the maturation drops were 0.01 U/ml, 0.02 U/ml, 1 µg/ml and 10% respectively. In the control group, no IL-7 was supplemented in maturation drops. In the treatment groups, maturation drops were supplemented with 0.5, 1, 2, 5 or 10 ng/ml of IL-7. COC were incubated in the maturation drops for 24 h at 38.5 °C in a CO2 incubator (5% CO2 in a humidified environment).

2.1.2. Assessment of oocyte maturation

Oocyte maturation rate in each experimental group was assessed following the 24 h of IVM. All COC were collected from culture drops, placed in a 1.5 ml micro centrifuge tube containing 150 µl of aspiration medium supplemented with hyaluronidase (6000 U/ml), incubated for 5 min at 37 °C, vortexed for 5 min and centrifuged at low speed briefly. The entire content of the tube was emptied in a 35 mm culture dish containing 2 ml of aspiration medium. The denuded oocytes were collected, washed in aspiration medium and observed under a stereo zoom microscope for the formation of polar body. The oocytes with visible polar body were considered as matured.

2.2. Assessment of intracellular ROS level

The effect of IL-7 supplementation on the production of ROS in ovine COC was assessed in this experiment. COC were cultured *in vitro* for 24 h in IVM medium without (control) or with 1 and 5 ng/ml of IL-7 supplementation (treatments). At the end of IVM, 10 COC from each experimental group were stained with 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to assess the intracellular ROS level. The stained COC were observed under a fluorescent microscope and the DCFH-DA staining pattern representing intracellular ROS level was compared among the control and treatment groups. The experiment was conducted in triplicates.

2.2.1. Collection of COC and IVM

COC were collected from abattoir-derived sheep ovaries and cultured in IVM drops for 24 h according to the procedures described previously in the Section 2.1.1. In the control group, no IL-7 was supplemented in maturation drops. In the treatment groups, maturation drops were supplemented with 1 or 5 ng/ml of IL-7.

2.2.2. COC staining for assessing intracellular ROS

The intracellular ROS level in COC was assessed according to a method described previously [13]. Briefly, cumulus expanded COC were collected from the control and treatment groups following the 24 h of IVM, washed in PBS and incubated in 10 µM of DCFH-DA in PBS for 30 min in dark. Following the incubation, the COC were washed thoroughly in PBS, placed into the well of a hanging drop slide in 20 µl of PBS and observed under a fluorescent microscope (Eclipse-80i Nikon instruments corporation, Tokyo, Japan) using FITC filter. Fluorescence as well as phase contrast (shifting the condenser wheel a little towards right side) images of the stained COC were precisely taken using the same parameters for all the groups.

2.3. IVM of COC with IL-7 and superoxide dismutase (SOD)-inhibitor

The effect of the supplementation of IL-7 and SOD-inhibitor (sodium diethyldithiocarbamate trihydrate, DETC) on maturation of ovine COC was assessed in this experiment. COC were cultured *in vitro* for 24 h in IVM medium without (control) or with 1 ng/ml of IL-7, 1 µM DETC and 1 ng/ml of IL-7 along with 1 µM DETC (treatments). Oocyte maturation rate was assessed at the end of IVM and compared among the control

and treatment groups. The experiment was conducted in triplicate. The total number of COC cultured and evaluated for maturation were 113, 115, 107 and 112, respectively in the control, 1 ng/ml of IL-7, 1 μM DETC and 1 ng/ml of IL-7 along with 1 μM DETC groups.

2.3.1. Collection of COC, IVM and assessment of oocyte maturation

COC were collected from abattoir-derived sheep ovaries and cultured in IVM drops for 24 h according to the procedures described previously in the Section 2.1.1. In the control group, no IL-7 or DETC was supplemented in IVM drops. In the treatment groups, IVM drops were supplemented with IL-7 (1 ng/ml), DETC (1 μM) or IL-7 (1 ng/ml) along with DETC (1 μM). Oocyte maturation rate in each experimental group was assessed following the 24 h of IVM according to the procedures described previously in the Section 2.1.2.

2.3.2. Selection of DETC dose

To find out a suitable dose of DETC for the current study, initially a dose-dependent experiment was conducted. IVM drops were supplemented with different concentrations of DETC (0.25, 0.50, 1 and 25 μM) in the treatment groups. No DETC was supplemented in IVM drops in the control group. COC (N = 30 in each group) were cultured in the IVM drops for 24 h and, maturation rate was determined at the end of IVM and compared among the treatment and control groups. Based on the results of this experiment, the specific dose of DETC (1 μM) was selected.

2.4. IVM of COC with IL-7 and NADPH-oxidase (NOX)-inhibitor

The effect of the supplementation of IL-7 and NOX-inhibitor (diphenyleneiodonium chloride, DPI) on maturation of ovine COC was assessed in this experiment. COC were cultured *in vitro* for 24 h in IVM medium without (control) or with 5 ng/ml of IL-7, 25 nM DPI and 5 ng/ml of IL-7 along with 25 nM DPI (treatments). Oocyte maturation rate was assessed at the end of IVM and compared among the control and treatment groups. The experiment was conducted in triplicate. The total number of COC cultured and evaluated for maturation were 111, 105, 110 and 109, respectively in the control, 5 ng/ml of IL-7, 25 nM DPI and 5 ng/ml of IL-7 along with 25 nM DPI groups.

2.4.1. Collection of COC, IVM and assessment of oocyte maturation

COC were collected from abattoir-derived sheep ovaries and cultured in IVM drops for 24 h according to the procedures described previously in the Section 2.1.1. In the control group, no IL-7 or DPI was supplemented in IVM drops. In the treatment groups, IVM drops were supplemented with IL-7 (5 ng/ml), DPI (25 nM) or IL-7 (5 ng/ml) along with DPI (25 nM). Oocyte maturation rate in each experimental group was assessed following the 24 h of IVM according to the procedures described previously in the Section 2.1.2.

2.4.2. Selection of DPI dose

To find out a suitable dose of DPI for the current study, initially a dose-dependent experiment was conducted. IVM drops were supplemented with different concentrations of DPI (12.5, 25, 50 and 100 nM) in the treatment groups. No DPI was supplemented in IVM drops in the control group. COC (N = 30 in each group) were cultured in the IVM drops for 24 h and, maturation rate was determined at the end of IVM and compared among the treatment and control groups. Based on the results of this experiment, the specific dose of DPI (25 nM) was selected.

2.5. Gene expression analyses

The effect of IL-7 supplementation on the expression of selected genes in ovine COC was assessed in this experiment. IVM medium was supplemented with 1 or 5 ng/ml of IL-7 (treatments) and relative gene expressions in COC were determined following the 6 and 24 h of IVM as compared to the control (no IL-7 supplementation). Expression analysis was performed by qPCR for 21 selected genes that were related to different functions such as mtDNA synthesis: nuclear respiratory factor-1 (*NRF1*) and mitochondrial transcription factor A (*TFAM*); ROS production and antioxidant enzymes: *NOX4*, *SOD1*, *SOD2*, *SOD3* and *Catalase*; apoptosis: Fas cell surface death receptor (*FAS*), *Caspase3*, *Caspase7*, *Caspase9*, *Caspase8*, apoptotic protease-activating factor 1 (*APAF1*), tumour protein P53 (*P53*), BCL2-associated X protein (*BAX*), B-cell CLL/lymphoma 2 (*BCL2*) and heat shock protein 70 (*HSPA1A*); and cell proliferation: phosphoinositide-3-kinase regulatory subunit 1 (*PI3KR1*), AKT serine/threonine kinase 1 (*AKT1*), BTG anti-proliferation factor 1 (*BTG1*) and *IL7RA*. The expression of the targeted genes was assessed in single COC obtained from each experimental group and the experiment was conducted in four replicates.

2.5.1. Sample collection, RNA isolation and cDNA synthesis

COC were collected from abattoir-derived sheep ovaries and cultured in IVM drops for 6 or 24 h according to the procedures described previously in the Section 2.1.1. In the control group, no IL-7 was supplemented in IVM drops. In the treatment groups, IVM drops were supplemented with 1 or 5 ng/ml of IL-7. Following the 6 and 24 h of culturing, COC were collected from IVM drops, washed in aspiration medium and evaluated under a stereo zoom microscope. The COC with nearly similar cumulus mass were collected and individual COC were placed into 1.5 ml micro centrifuge tubes containing 100 μl of TRI reagent. The tubes were stored at −80 °C until processed for RNA isolation.

The extraction of RNA from COC using TRI reagent was performed following the manufacturer's instructions and linear acrylamide (Life Technologies Corporation, NY, USA) was used for effective precipitation of RNA. The extracted total RNA was dissolved in nuclease-free water and subjected to RNase-free DNase I (Life Technologies Corporation, NY, USA) treatment following the manufacturer's instructions. Following the DNase treatment, the RNA pellet was washed, air dried, dissolved in 10 μl of nuclease-free water and immediately reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) following the manufacturer's instructions. The synthesized cDNA was stored at −20 °C until used for gene expression analysis by qPCR.

2.5.2. Determining gene expression by qPCR

The qPCR analysis of the selected genes was performed in a Quant Studio® 3 qPCR System (Thermo Fisher Scientific, CA, USA) using SsoFast™ Eva Green® Supermix (Bio-Rad Laboratories Inc, CA, USA). Briefly, 1 μl of diluted cDNA (1:1 with nuclease free water), 0.25 μM of each primer and 1X Eva Green mix were used in a total volume of 10 μl. The qPCR conditions were as follows: initial denaturation at 95 °C for 3 min and 40 cycles of 95 °C for 15 sec and annealing temperature for 30 sec. The identity of amplified product was confirmed by agarose gel electrophoresis and melting curve analysis (continuous from 65 to 95 °C; increment 0.15 °C/sec). The details of the selected genes and the primer pairs used in the study are provided in Table 1 [14–26]. The relative changes in gene expression were determined using $2_T^{-\Delta\Delta C}$ method [27]. ACTB [17] and 18 s [18] were used as endogenous reference gene and the geometric mean of their Ct values was used for calculating relative gene expression level [28]. Expression values of the genes within each IL-7 concentration were compared among the control, 6 h-treatment and 24 h-treatment groups.

2.6. Statistical analysis

Statistical analyses were performed using the PASW 18.0.0 software package (SPSS/IBM, IL, USA). The values expressed in percentage for maturation rate were subjected to arcsine transformation to maintain homogeneity of variances [18] and general linear model (GLM) procedure was followed to analyze the variations in maturation rates among the different experimental groups. The values expressed in fold

Table 1
Details of the selected genes and primer pairs used for gene expression analyses.

Gene	Primer sequences (5′-3′)	Reference/Genbank accession no	Annealing temperature (^{0}c)	product size	Amplification efficiency %, (R^2)
TFAM	F: CAGACTGGCAGGTGTACA; R: CGAGGTCTTTTTGGTTTTCCA	[14]	55	163	90 (0.99)
SOD1	F: TCATGGGTTCCACGTCCAT; R: GAGGGCCTGCACTGGTACAG	[15]	60	62	93 (0.99)
SOD2	F: CAGGATCCCCTGCAAGGA; R: CATGCTCCCACACGTCAATC	[15]	60	64	96 (0.99)
SOD3	F: TCACCTTGATTTTTCTCCTTCCTT; R: TGGCAGAAGTGGTACTCCAGAGT	[15]	60	64	95 (0.99)
NOX4	F: GCTGGAGGCATTGGAGTCA; R: TTTCCAGTCATCCAGCAGAGTGT	[15]	55	63	90 (0.99)
Catalase	F: GCTCCAAATTACTACCCCAATAGC; R: GCACTGTTGAAGCGCTGTACA	[16]	60	104	90 (0.99)
Caspase3	F: TAGCAAGTTTCTTCAGAGGG; R: GTCTCAATACCACAGTCCAG	[17]	60	106	95 (0.99)
BAX	F: CATGGAGCTGCAGAGGATGA; R: GTTGAAGTTGCCGTCGGAAA	[18]	60	100	98 (0.99)
P53	F: GGAAGAATCGCAGGCAGAACT; R: GGAGAGCTCGGAGGACAGAA	[17]	60	109	101 (0.99)
Caspase7	F: GAATGGGTGTCCGCAACG; R: TTGGCACAAGAGCAGTCGTT	[19]	60	106	91 (0.99)
Caspase9	F: TTCCCAGGTTTTGTTTCCTG; R: CCTTTCACCGAAACAGCATT	[20]	55	143	90 (0.99)
Caspase8	F: CATCCAGTCACTTTGCCAGA; R: GCATCTGTTTCCCCATGTTT	[20]	55	128	91 (0.99)
FAS	F: CTGGAGCAAGTTCCTGCCAA; R: CTCCGTCGCGTTTGC	[21]	55	105	91 (0.99)
APAF1	F: AAGGTGGAGTACCACAGAGG; R: TCCATGTATGGTGACCCATCC	[22]	60	116	98 (0.99)
HSPA1A	F: CTGATCAAGCGCAACTCCA; R: AGCAGGTTGTTGTCCCGAGT	[18]	60	131	99 (0.99)
NRF1	F: CCCAAACTGAGCACATGGC; R: GTTAAGTATGTCTGAATCGTC	[23]	60	161	94 (0.99)
PI3KR1	F: AAGAACAATGCCAAACCCAGGAGC; R: CCTGCTTCTTCAAGTCTTCTTCCAACC	[24]	55	174	91 (0.99)
BTG1	F: TGCAGGAGCTGCTGGCAG; R: TGCTACCTCCTGCTGGTGA	[25]	60	270	91 (0.99)
IL-7RA	F: ACCAAGCTGACACTCCTAC; R: CCATTCACTCCAGAAGCC	[18]	60	108	97 (0.99)
18s	F: AGAAACGGCTACCACATCCAA; R: CCTGTATTGTTATTTTTCGT	[18]	60	90	96 (0.99)
ACTB	F: CGCAGACAGGATGCAGAAAG; R: GCTGATCCACATCTGCTGGA	[26]	60	148	93 (0.99)
BCL2	F: GCGGCCCCTGTTTGATTTC; R: TTATGGCCCAGATAGGCACCC	This study (XM_018039337.1)	55	99	92 (0.99)
AKT1	F: TTCAAGCCTCAGGTCACGTC; R: GTCTTGGTCAGGTGGCGTAA	This study (NM_001161857.1)	55	93	99 (0.99)

change for gene expression were subjected to log2 transformation to maintain homogeneity of variances [18] and GLM procedure was followed to analyze the variations in gene expressions among the different experimental groups within each IL-7 concentration. All pair wise multiple-comparisons between means following GLM were performed using the Duncan's multiple-range test. The data are presented as mean ± SD and differences were considered significant if the P value was less than 0.05.

3. Results

3.1. Effect of IL-7 on IVM of ovine COC

The effect of IL-7 supplementation on maturation of COC is depicted in Fig. 1. Oocyte maturation rates were compared among the control and IL-7 supplemented groups. IL-7 supplementation at a concentration of 0.5 ng/ml did not improve oocyte maturation as compared to the control. Nevertheless, the maturation rate (%) was found significantly (P = 0.000) greater with the 1 ng/ml of IL-7 supplementation (69.5) than control (60.0). In contrast, the maturation rate was reduced significantly (P = 0.000) with the 2 (47.1), 5 (39.2) and 10 ng/ml (39.1) of IL-7 supplementation as compared to the control.

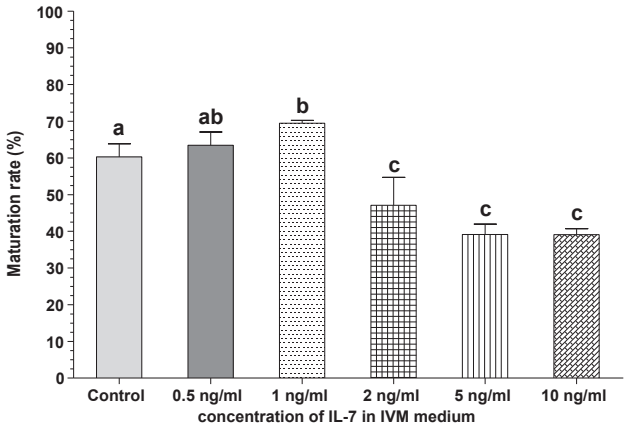

Fig. 1. Effect of different doses of IL-7 supplementation on *in vitro* maturation rate (mean ± SD) of ovine cumulus-oocyte complexes (COC). Means were compared among the control and treatments using the general linear model procedure followed by the Duncan's multiple-range test. a – c: Values without a common superscript above error bars differ significantly (P = 0.000) from each other and the values with a common superscript above error bars are not significantly different from each other. Total number of COC cultured and evaluated for maturation were 131, 126, 127, 122, 124 and 128, respectively in the control and 0.5, 1, 2, 5, and 10 ng/ml of IL-7 supplemented groups.

3.2. Effect of IL-7 on ROS generation in ovine COC

The extent of ROS generation in matured COC was assessed based on the DCFH-DA staining and representative photographs are shown in Fig. 2. The DCFH-DA staining patterns were compared among the control and IL-7 supplemented groups. The intensity of DCFH-DA fluorescence in the COC treated with 1 ng/ml of IL-7 was found marginally greater than the control. In contrast, intense DCFH-DA fluorescence was observed in the COC treated with 5 ng/ml of IL-7 as compared to the control or 1 ng/ml of IL-7. The results indicated that the 1 ng/ml of IL-7 treatment marginally increased ROS production as compared to the control. In contrast, the 5 ng/ml of IL-7 treatment considerably increased ROS production as compared to the control or 1 ng/ml of IL-7 treatment.

3.3. Effect of IL-7 and SOD-inhibitor on IVM of ovine COC

Supplementation of 0.25, 0.50, 1 and 25 μM of DETC (SOD-inhibitor) in IVM medium reduced oocyte maturation rate (%) as compared to the control and the respective values were found 60.0, 56.7,

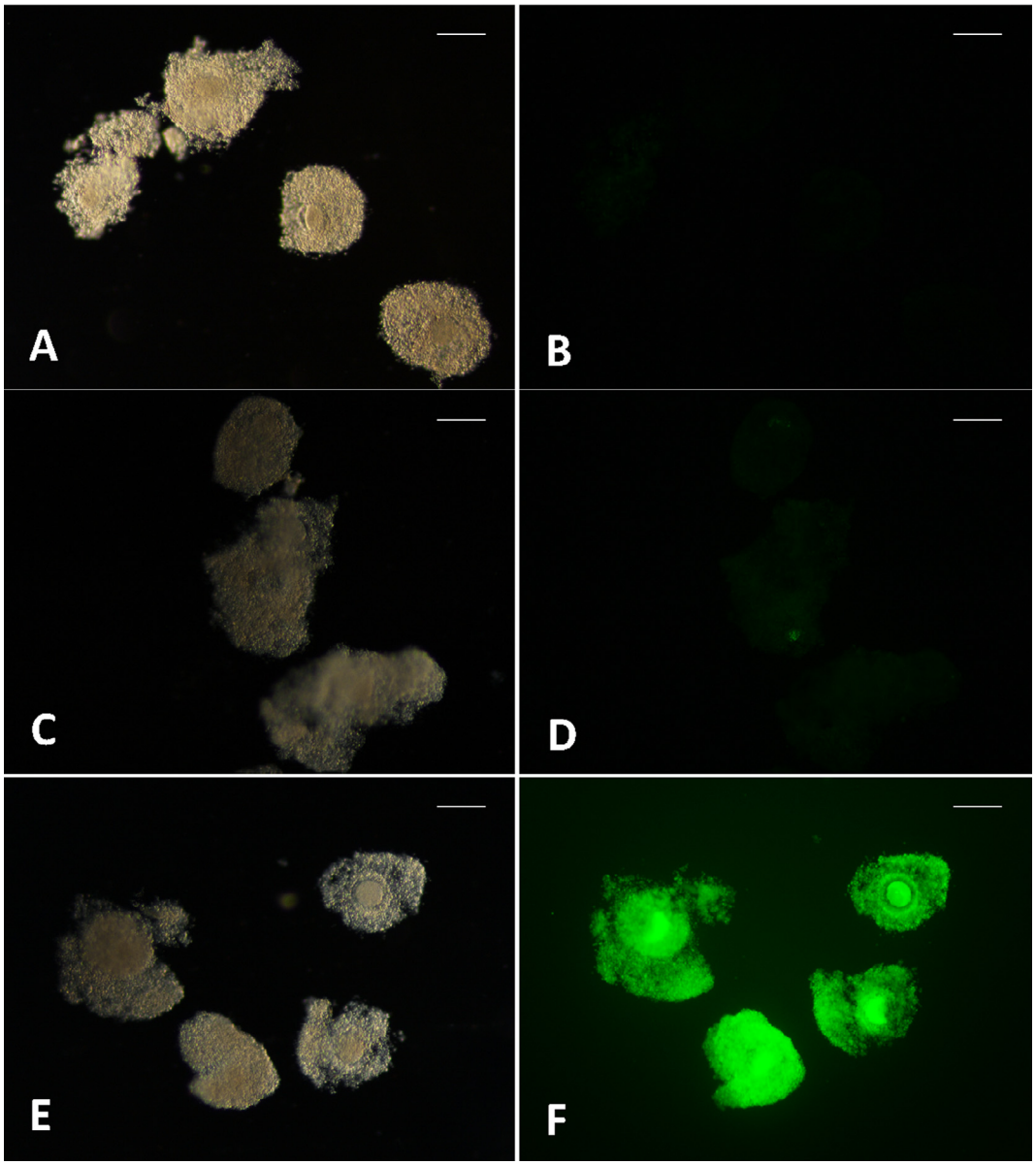

Fig. 2. Effect of IL-7 supplementation on the production of reactive oxygen species (ROS) in ovine cumulus-oocyte complexes (COC). COC were matured *in vitro* for 24 h without (control) or with IL-7 (1 or 5 ng/ml) supplementation and matured COC were stained with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to detect the intracellular level of ROS. A: phase-contrast, control; B: DCFH-DA fluorescence, control; C: phase-contrast, 1 ng/ml IL-7; D: DCFH-DA fluorescence, 1 ng/ml IL-7; E: phase-contrast, 5 ng/ml IL-7; F: DCFH-DA fluorescence, 5 ng/ml IL-7; Bar = 200 μm.

50.0, 16.7 and 63.3. Based on these results, 1 μM of DETC was selected for SOD inhibition subsequently. Oocyte maturation rates were compared among the control and DETC, IL-7 and DETC + IL-7 supplemented groups (Fig. 3). The IL-7 (1 ng/ml) treatment significantly (P = 0.003) improved oocyte maturation rate (69.1%) as compared to the control (63.6%). In contrast, the treatment of COC with the combination of IL-7 (1 ng/ml) and SOD-inhibitor (1 μM DETC) significantly (P = 0.003) reduced the maturation rate (57.0%) as compared to the treatment of IL-7 (1 ng/ml) alone and it was comparable with that of the control.

3.4. Effect of IL-7 and NOX-inhibitor on IVM of ovine COC

Supplementation of 25, 50 and 100 nM of DPI (NOX-inhibitor) in IVM medium reduced oocyte maturation rate (%) as compared to the control or supplementation of 12.5 nM of DPI and the respective values were found 46.7, 36.7, 33.3, 60.0 and 60.0. Based on these results, 25 nM of DPI was selected for inhibiting NOX-mediated ROS production subsequently. Oocyte maturation rates were compared among the control and DPI, IL-7 and DPI + IL-7 supplemented groups (Fig. 4). The IL-7 (5 ng/ml) treatment significantly (P = 0.000) reduced oocyte maturation rate (42.7%) as compared to the control (61.5%). In contrast, the treatment of COC with the combination of IL-7 (5 ng/ml) and NOX-inhibitor (25 nM DPI) significantly (P = 0.000) improved the maturation rate (55.0%) as compared to the treatment of IL-7 (5 ng/ml) alone and it was comparable with that of the control.

3.5. Effect of IL-7 on gene expression in ovine COC

The effect of supplementation of different concentrations of IL-7 (1 and 5 ng/ml) on the expression pattern of the genes related to mtDNA synthesis (*NRF1* and *TFAM*), ROS production and antioxidant enzymes (*NOX4*, *SOD1*, *SOD2*, *SOD3* and *Catalase*), apoptosis (*FAS*, *Caspase3*, *Caspase7*, *Caspase9*, *Caspase8*, *APAF1*, *P53*, *BAX*, *BCL2* and *HSPA1A*)

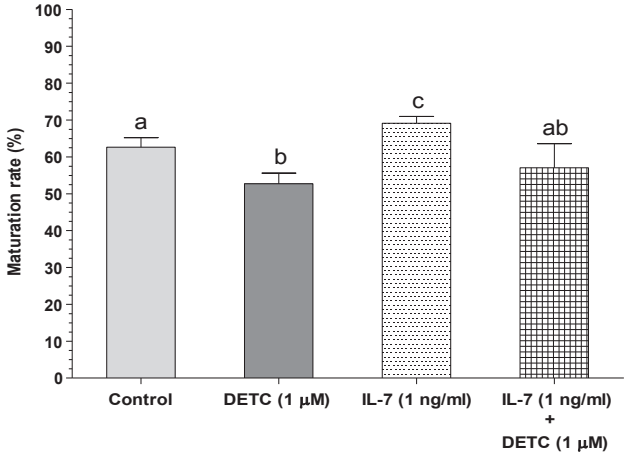

Fig. 3. Effect of supplementation of IL-7 and SOD-inhibitor (sodium diethyldithiocarbamate trihydrate, DETC) on *in vitro* maturation rate (mean ± SD) of ovine cumulus-oocyte complexes. Means were compared among the control and treatments using the general linear model procedure followed by the Duncan's multiple-range test. a – c: Values without a common superscript above error bars differ significantly (P = 0.003) from each other and the values with a common superscript above error bars are not significantly different from each other. Total number of COC cultured and evaluated for maturation were 113, 107, 115 and 112, respectively in the control, DETC (1 μM), IL-7 (1 ng/ml) and IL-7 (1 ng/ml) + DETC (1 μM) groups.

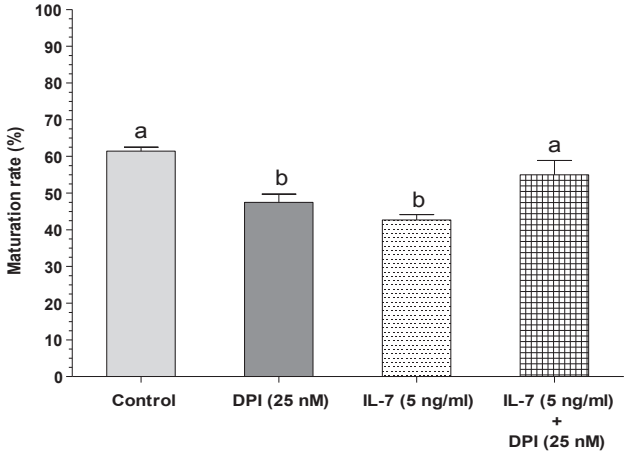

Fig. 4. Effect of supplementation of IL-7 and NOX-inhibitor (diphenyleneiodonium chloride, DPI) on *in vitro* maturation rate (mean ± SD) of ovine cumulus-oocyte complexes. Means were compared among the control and treatments using the general linear model procedure followed by the Duncan's multiple-range test. a – b: Values without a common superscript above error bars differ significantly (P = 0.000) from each other and the values with a common superscript above error bars are not significantly different from each other. Total number of COC cultured and evaluated for maturation were 111, 110, 105 and 109, respectively in the control, DPI (25 nM), IL-7 (5 ng/ml) and IL-7 (5 ng/ml) + DPI (25 nM) groups.

and cell proliferation (*PI3KR1*, *AKT1*, *BTG1* and *IL7RA*) at different stages of IVM (6 and 24 h) is depicted in Fig. 5. Within each IL-7 concentration (1 or 5 ng/ml), expression values of the genes were compared among the control, 6 h-treatment and 24 h-treatment groups.

3.5.1. Effect of 1 ng/ml IL-7 on gene expression

No significant difference in the expression of *NOX4, SOD1*, *SOD2*, *SOD3*, *Catalase*, *FAS*, *Caspase8*, *P53*, *BAX*, *BCL2*, *PI3KR1*, *AKT1*, *BTG1* and *IL7RA* was observed at the 6 h of IVM with the supplementation of 1 ng/ml of IL-7 as compared to the control. In contrast, at the 6 h of IVM, significantly upregulated expression (fold change) of *NRF1* (1.28, P = 0.000), *TFAM* (1.60, P = 0.000), *Caspase3* (1.66, P = 0.007) and *Caspase9* (1.46, P = 0.000) and, significantly downregulated expression of *Caspase7* (0.79, P = 0.002), *APAF1* (0.47, P = 0.000) and *HSPA1A* (0.59, P = 0.011) were observed with the 1 ng/ml of IL-7 supplementation as compared to the control.

At the 24 h of IVM, no significant difference in the expression of *NOX4, SOD1*, *FAS*, *Caspase3, Caspase8*, *APAF1*, *PI3KR1, AKT1* and *BTG1* was observed with the 1 ng/ml of IL-7 supplementation as compared to the control. In contrast, significantly upregulated expression of *SOD2* (1.57, P = 0.003), *SOD3* (1.64, P = 0.004) and *Catalase* (1.25, P = 0.012) and, significantly downregulated expression of *NRF1* (0.76, P = 0.000), *TFAM* (0.68, P = 0.000), *Caspase7* (0.60, P = 0.002), Caspase9 (0.63, P = 0.000), *P53* (0.62, P = 0.009), *BAX* (0.74, P = 0.023), *BCL2* (0.55, P = 0.001), *HSPA1A* (0.51, P = 0.011) and *IL7RA* (0.44, P = 0.030) were observed at the 24 h of IVM with the 1 ng/ml of IL-7 supplementation as compared to the control.

Significantly greater expression of *SOD2* (P = 0.003), *SOD3* (P = 0.004), *Catalase* (P = 0.012), *APAF1*(P = 0.000) and *BTG1* (P = 0.022) and, significantly lesser expression of *NRF1* (P = 0.000), *TFAM* (P = 0.000), *Caspase3* (P = 0.007), *Caspase7* (P = 0.002), *Caspase9* (P = 0.000), *P53* (P = 0.009), *BAX* (P = 0.023) and *BCL2* (P = 0.001) were observed at the 24 as compared to 6 h of IVM within the 1 ng/ml IL-7 group. In contrast, the expression of *NOX4*, *SOD1*, *FAS*, *Caspase8*, *HSPA1A*, *PI3KR1*, *AKT1* and *IL7RA* did not differ significantly between the 6 and 24 h of IVM within the 1 ng/ml IL-7 group.

3.5.2. Effect of 5 ng/ml IL-7 on gene expression

The expression of *SOD2*, *SOD3*, *FAS*, *Caspase7*, *Caspase9*, *P53*, *BAX*, *BCL2*, PI3KR1 and *BTG1* did not differ significantly with the 5 ng/ml of IL-7 supplementation as compared to the control at the 6 h of IVM. In contrast, at the same time point, significantly upregulated expression of *NRF1* (1.36, P = 0.047), *TFAM* (1.37, P = 0.005), *NOX4* (1.30, P = 0.001), *Catalase* (1.48, P = 0.001), *Caspase3* (2.10, P = 0.020) and *AKT1* (1.47, P = 0.006) and, significantly downregulated expression of *SOD1* (0.52, P = 0.010), *Caspase8* (0.32, P = 0.001), *APAF1* (0.40, P = 0.000), *HSPA1A* (0.56, P = 0.014) and *IL7RA* (0.67, P = 0.000) were observed with the 5 ng/ml of IL-7 supplementation as compared to the control.

The expression of *NRF1*, *SOD1*, *SOD2*, *Caspase3*, *Caspase7*, *Caspase9*, *Caspase8*, *P53* and *HSPA1A* was not significantly different at the 24 h of IVM with the 5 ng/ml of IL-7 supplementation as compared to the control. In contrast, significantly upregulated expression of *TFAM* (1.36, P = 0.005), *NOX4* (1.98, P = 0.001), *FAS* (1.38, P = 0.048), *APAF1* (2.62, P = 0.000), *BAX* (1.31, P = 0.021), *BCL2* (1.64, P = 0.046), *AKT1* (1.29, P = 0.006) and *BTG1* (1.62, P = 0.028) and, significantly downregulated expression of *SOD3* (0.59, P = 0.013), *Catalase* (0.68, P = 0.001), *PI3KR1* (0.59, P = 0.043) and *IL7RA* (0.39, P = 0.000) were observed at the 24 h of IVM with the 5 ng/ml of IL-7 supplementation as compared to the control.

No significant difference in the expression of *NRF1*, *TFAM*, *SOD2*, *SOD3*, *FAS*, *Caspase3*, *Caspase7*, *Caspase9*, *P53*, *HSPA1A*, and *AKT1* was observed between the 6 and 24 h of IVM within the 5 ng/ml IL-7 group. In contrast, significantly greater expression of *NOX4* (P = 0.001), *SOD1* (P = 0.010), *Caspase8* (P = 0.001), *APAF1* (P = 0.000), *BAX* (P = 0.021), *BCL2* (P = 0.046) and *BTG1* (P = 0.028) and, significantly lesser expression of *Catalase* (P = 0.001), *PI3KR1* (P = 0.043) and *IL7RA* (P = 0.000) were observed at the 24 as compared to 6 h of IVM within the 5 ng/ml IL-7 group.

4. Discussion

The IL-7 mediated signals are linked to the development, survival and cell cycle progression. Nevertheless, the mechanism of its involvement in oocyte maturation process is not well characterized. In the current study, we investigated the effect of IL-7 supplementation in IVM medium on the maturation rate, ROS production and gene expression of

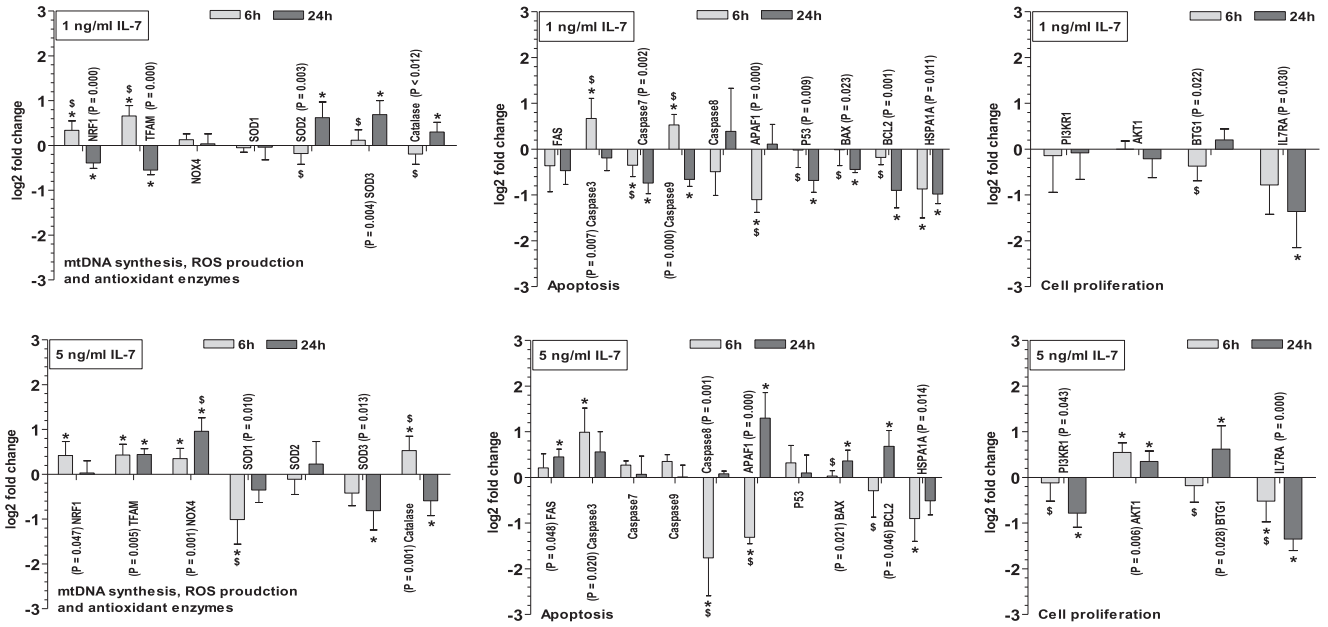

Fig. 5. Relative expression (mean ± SD) of the targeted genes in ovine cumulus-oocyte complexes (COC) with the 1 and 5 ng/ml of IL-7 supplementation (treatments) as compared to the control (no IL-7 supplementation) at the 6 and 24 h of *in vitro* maturation. Expressions of the genes were determined in single COC and the experiment was conducted in four replicates. Within each IL-7 concentration (1 or 5 ng/ml), means were compared among the control, 6 h-treatment and 24 h-treatment groups using the general linear model procedure followed by the Duncan's multiple-range test. * indicates a significant difference between the control and treatments (1 or 5 ng/ml IL-7) at the 6 or 24 h of IVM. $ indicates a significant difference between the 6 and 24 h of IVM within a treatment (1 or 5 ng/ml IL-7). P values are indicated in parentheses for the significantly different means.

ovine COC. The results indicate that the maturation rate, intracellular ROS production and gene expression signature of ovine COC were significantly influenced by the supplementation of IL-7 in a dose dependent manner.

Previous reports indicate that IL-7 promotes survival and replication of granulosa cells, germinal vesicle breakdown and quality and nuclear maturation of preovulatory oocytes in rat and mice [10–12]. Although exogenous IL-7 is beneficial for cell survival, at high concentration, IL-7 may trigger apoptosis [6]. It was evident in the current study that the 1 ng/ml of IL-7 supplementation enhanced the maturation of ovine COC significantly. Nevertheless, IL-7 was detrimental for the process, when supplemented at higher concentrations (> 1 ng/ml). Based on the results of this experiment, two concentrations of IL-7 (beneficial: 1 ng/ml; detrimental: 5 ng/ml) were selected for conducting the further experiments for understanding the underlying mechanisms of IL-7 involvement in oocyte maturation.

The involvement of ROS in ovulation, fertilization and conception is suggested [9]. Low concentration of ROS is beneficial for meiotic resumption during oocyte maturation, but high ROS level is associated with meiotic cell cycle arrest and apoptosis [9]. It was evident that the supplementation of 1 ng/ml of IL-7 was associated with a marginal increase in intracellular ROS level in the matured oocytes. In contrast, substantially greater intracellular ROS level was observed in the matured oocytes, when 5 ng/ml of IL-7 was supplemented. We further noticed that the 1 ng/ml of IL-7 did not stimulate oocyte maturation, when cellular antioxidant mechanisms were disrupted by supplementing SOD-inhibitor. On the contrary, the detrimental effect of 5 ng/ml of IL-7 could be nullified by hindering the process of intracellular ROS generation with NOX-inhibitor. The results indicate that 1 ng/ml of IL-7 exerted its beneficial effect on oocyte maturation by maintaining a favourable level of intracellular ROS. Conversely, the detrimental effect of 5 ng/ml of IL-7 on oocyte maturation was mediated through the excessive production of intracellular ROS causing oxidative damage.

TFAM and *NRF1* are known to regulate the synthesis of mtDNA, which is important for oocyte maturation [29,30]. The mtDNA-mediated production of ATP and ROS through oxidative phosphorylation are essential for cell proliferation, oocyte maturation and the early embryogenesis [9,14,31–33]. The current study revealed that the supplementation of IL-7 significantly influenced the expression of *TFAM* and *NRF1* in a dose and time dependant manner. Significantly greater expression of *TFAM* and *NRF1* at the 6 h of IVM in the IL-7 supplemented groups (1 and 5 ng/ml) indicates that both concentrations of IL-7 likely stimulated the synthesis of mtDNA during the early maturation stage. In contrast, at the late maturation stage, mtDNA synthesis was likely stimulated by the supplementation of 5 ng/ml of IL-7 as evident from the sustained upregulated expression of *NRF1* at the 24 h of IVM in this group.

NOX4 belongs to the enzyme family NOX and is responsible for the synthesis of ROS. Previous evidences indicate that NOX4 is localized to mitochondria, wherein it contributes to the NOX-dependent mitochondrial oxidative stress [34]. Significantly greater expression of *NOX4* was found at the 6 and 24 h of IVM with the 5 ng/ml, but not with the 1 ng/ml of IL-7 supplementations. The results indicate that the 5 ng/ml of IL-7 likely stimulated the NOX4-mediated ROS generation during the early and late stages of oocyte maturation.

Antioxidants are strongly involved in oocyte maturation and a disruption in the balance between ROS and antioxidant defence systems can cause aberrant oocyte maturation [35]. SOD1, SOD2, SOD3 and Catalase are important antioxidant enzymes in most cells and they are involved in controlling the elevated level of ROS during IVM of oocyte [36]. In the current study, the 1 ng/ml of IL-7 treatment resulted in a significantly upregulated expression of these genes except *SOD1* at the 24 h of IVM. The results indicate the likely existence of a favourable antioxidant mechanism in the COC of this group. In contrast, antioxidant mechanism of the COC was possibly compromised with the supplementation of 5 ng/ml of IL-7 as significantly downregulated expression of *SOD1* (6 h), *SOD3* (24 h) and *Catalase* (24 h) was observed in this group.

Although IL-7-induced ROS generation promotes cell survival [7], accumulation of ROS inside the cell is often observed in the progress of apoptosis and can be an indication of the process [8]. The existence of

intracellular caspase activity is a definitive confirmation of cellular apoptosis [37]. BCL2 blocks apoptosis by modulating the mitochondrial release of cytochrome-c causing the interaction of APAF1 with Caspase9 and by binding to BAX [37,38]. P53 induced apoptosis requires the generation of ROS [39] and transcriptional activation of *BAX* and *FAS* [37,40]. *HSPA1A* is an antiapoptotic gene and it is involved in rescuing cells from apoptosis later in the death signalling pathway [41,42]. It was evident that the IL-7 supplementation affected the expression of these genes in COC in a dose and time dependant manner. Similar or significantly lesser expression of these genes at the completion of IVM (24 h) with the 1 ng/ml of IL-7 supplementation indicates a downregulated apoptotic process in the matured COC in this group. In contrast, similar or significantly greater expression of these genes at the 24 h with the 5 ng/ml of IL-7 treatment indicates a stimulated apoptotic process in the matured COC in this group.

Previous report indicates that IL-7 activates PI3K/Akt pathway, which is a major effector of IL-7 induced viability, growth and proliferation of cells [7]. PI3K/Akt pathway is the well documented downstream signalling of NOX and a positive feedback loop between NOX4 and PI3K/Akt signalling is reported [43]. The transcription factor BTG1 inhibits cellular proliferation [44] and it is involved in the irreversible exit from the cell cycle leading towards differentiation [45]. The supplementation of 1 ng/ml of IL-7 did not influence the expression of *PI3KR1*, *AKT1* and *BTG1* at the 6 or 24 h of IVM as compared to the control. In contrast, significantly upregulated expression of *AKT1* was observed with the 5 ng/ml of IL-7 supplementation at the 6 and 24 h of IVM as compared to the control. The expression of *NOX4* was also found significantly greater at both the time points in the same treatment group. The results indicate that the AKT signalling was likely stimulated through the greater *NOX4* expression with the 5 ng/ml of IL-7 supplementation. A significantly upregulated expression of *BTG1* at the 24 h in the 5 ng/ml IL-7 group indicates that greater IL-7 dose exerted a negative effect on oocyte maturation through the activation of *BTG1*.

IL-7 binds to its specific receptor complex for exerting its pro-survival and proliferative effects on cells [46]. Feedback control is an important regulatory process in the IL-7 receptor signalling and it involves both positive and negative feedback loops [10,47]. It was evident that both concentrations of IL-7 (1 and 5 mg/ml) significantly downregulated the expression of *IL7RA* at the 24 h of IVM. The results indicate that a negative feedback loop was probably established between IL-7 and its receptor following the exposure of COC to exogenous IL-7 for an extended period.

5. Conclusion

In conclusion, the supplementation of IL-7 at low concentration significantly improved the maturation rate of ovine COC *in vitro*. The beneficial effects of the low IL-7 dose were likely mediated through the stimulation of mtDNA synthesis, favourable intracellular ROS level and cellular antioxidant mechanism and, downregulated apoptotic process. In contrast, greater concentrations of IL-7 were found detrimental for the maturation of ovine COC. The harmful effects of the higher IL-7 concentrations were possibly arbitrated through the ROS-mediated oxidative stress, compromised cellular antioxidant mechanism and stimulated apoptotic signalling.

Conflicts of interest

The authors declared that there is no conflict of interest.

Acknowledgements

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