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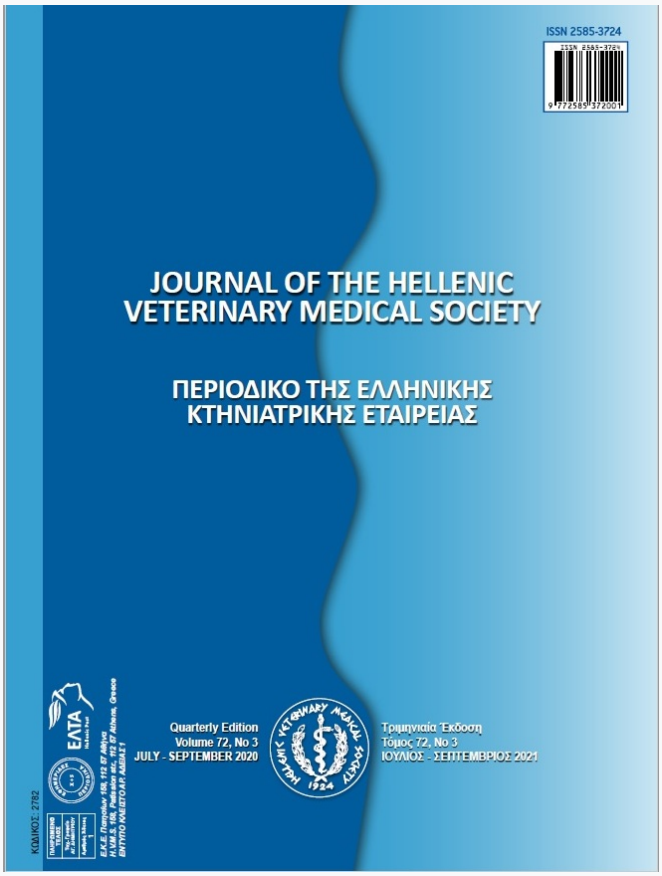

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The supplementation of ovine oocyte maturation medium with triiodothyronine affects the embryo development and apoptotic gene expression

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ABSTRACT: Triiodothyronine (T3) plays an essential role in different animal species' embryonic development. The present research was designed to identify the effect of triiodothyronine on the in vitro ovine embryonic development and the expression of apoptotic genes.A total of 436 immature cumulus-oocyte complexes (COCs) were cultured for 24 *h* in the oocyte maturation medium supplemented with two concentrations of T3 (T-10 and T-100 *ng/mL*) or without T3(T-0: control group). Oocyte maturation, cleavage, and blastocyst rates were assessed under an inverted microscope as crucial indicators of embryo development.The relative mRNA abundance of BCL-2-associated X protein (*BAX*) and anti-apoptotic B-cell lymphoma-2 (*BCL2*) were determined at blastocysts (day 8 after IVF on day 0)by quantitative reverse transcription PCR.The data were analyzed by logistic regression using the GLIMMIX procedure followed by Chi-Square, and one-way ANOVA tests. The higher concentration of T3(100 *ng/mL*) significantly decreased cumulus expansion and blastocyst rate compared to controls ($P<0.001$). Additionally, a significantly higher expression level of *BAX*($P<0.001$) and a dramatically lower expression level of *BCL2* ($P<0.01$) were detected in the T-100*ng/mL* group compared to the controls.However, the relative mRNA level of *BCL-2* was significantly higher in the T-10 *ng/mL* group compared to the control group ($P<0.01$).It appears that the supplementation of ovine oocyte maturation medium with T3 at high concentration (100 *ng/mL*) suppresses the ratio of blastocyst formation.

Keywords:Embryo, Gene expression,In vitro oocyte maturation,Triiodothyronine

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INTRODUCTION

One of the goals of improving in vitro maturation conditions is to achieve optimum production rate, in order to produce embryos that can be frozen or transferred (Hashimoto, 2009). Improvement of culture medium composition increases the number of blastocysts during oocyte maturation (Alsalim et al., 2018;Laskowski et al., 2017). The oocytes are entirely dependent on the biophysical conditions of the culture system(Gardner and Michelle,2003;Flood et al., 1993). In general, the in vitro culture of livestock oocyte is associated with reduced embryogenesis, potential and altered gene expression (Lane and Gardner, 2007). Several studies have documented the beneficial effects of enriching culture medium with hormones, such as growth hormones, melatonin, and leptin, which improve the efficiency of oocyte development(Arias-Alvarez et al.,2011;El-Raey et al.,2011;Pers-Kamczyc et al.,2010).In addition, it has been reported that the thyroid hormone (TH), triiodothyronine (T3), plays a key role in the embryogenesis of fish (Detlaf and Davydova, 1974) and birds (Sechman et al.,2009).In vitro studies in mammals have shown contradictory outcomes about the effects of T3 on the ovary, perhaps due to genetic variations or differences in THs doses (Komninou et al., 2016; Ashkar et al.,2010a). According to previous findings, triiodothyronine (T3) may reducethe FSH-driven aromatase activity in cumulus oophorus cells collected from infantile and adult rodents'follicles leading to a reduction in estradiol production (Cecconi et al.,1999). In another report, rodent follicles lost their ability to form an antrum when co-cultured with high doses of T3 and consequently, oocytes represented a poorer tendency to pass via meiosis beyond germinal vesicle breakdown (GVBD) (Vissenberg et al., 2015). The previous study showed that when bovine granulosa and theca cells were cultured in vitro (IVC) in media supplemented with T3 and T4, the production of steroids was increased. For instance, in the presence of luteinizing hormone and insulin, both THs could increase and rostenedione production by theca cells (Spicer et al.,2001). Several *in vivo* and *in vitro* studies have demonstrated that THs are involved in the induction and inhibition of programmed cell death through mitochondrial pathways (Shi et al., 2001; Asahara et al., 2003; Laoag-Fernandez et al., 2004). However, the effect of THs on oocyte maturation and blastocyst formation rates has not yet been established in sheep. It appears that THs are involved in the regulation of early embryogenesis and expression of various apoptotic genes;thus, it is assumed that THs may play a role in sheep embryo development. Therefore, this research aimed to evaluate the possible beneficial or detrimental effects of adding triiodothyronine to oocyte maturation medium on the ovine blastocyst formation as well as the expression of pro-apoptotic (*BAX*) and anti-apoptotic (*BCL-2*) genes.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis. MO, USA) or Gibco (Grand Island, NY, USA) unless otherwise stated. All protocols in this study were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, the University of Tabriz, with ethical code **(**IR.FVM.REC.1396.139).

Experimental design

To assess the effect of T3 on the meiosis process during IVM, the maturation medium was supplemented with exogenous triiodothyronine (Sigma cat No.709719) in concentrations of 10 or 100 *ng/mL.* COCs were randomly divided into three groups (T-0, T-10, T-100*ng/mL*).Each group was assessed for the stage of nuclear maturation after IVM. The number of blastocysts was recorded after IVF and IVC (day 8 - day 0: IVF).In addition, the expression of apoptosis-related genes was examined in the blastocysts obtained at the end of IVC period, to assess the effect of different concentrations of T3 on apoptosis.

Collection of ovine oocytes and in vitro maturation (IVM)

Ovine oocytes(*n*=450) were collected as described earlier(El-Raey et al.,2011). Initially, ovine ovaries were gathered from an abattoir and delivered to the research lab in normal saline supplemented with streptomycin (100*μg/mL*) and penicillin (100*IU/mL*) at 35-37°C within 2*h*. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles with a diameter of 2-6 mm using an18-gauge needle connected to 5-10 mL disposable syringe. HEPES-buffered tissue culture medium 199 (H-TCM199), complemented with 10%FBS, 0.2 mM sodium pyruvate, 100 µl/mL heparin and 5µg/mL gentamicin, was used for manipulation of the COCs before culture (Davachi et al., 2018). Only the oocytes with at least three layers of cumulus granulosa cells and homogeneous cytoplasm were used in three independent repeats, allocating about 50COCsper treatment (T-0, T-10 and T-100 ng/mL).After washing, the COCs were transferred to50

μLdrops of in vitro maturation medium(IVM)consisting of modified tissue culture medium (mTCM) supplemented with 10 or 100 ng/mL of T3 and 10 % FBS, 1mL glutamine, 2.5mM Na pyruvate, follicle stimulating hormone(FSH;*10μg/mL*),human menopausal gonadotropin (HMG;*0.075IU/mL*), estradiol17-β(*1μg/mL*) and,gentamycin sulfate (5 *μg/mL),*under mineral *oil* and cultured for 24 *h* under a maximum humidified atmosphere of5 % CO_2in the air, at 38.5°C.

Evaluation of oocyte maturation

The scale of cumulus cell expansion was subjectively measured by the stereomicroscope after 24*h* of IVM; the COCs were categorized as fully expanded(G1), partially expanded(G2) or not expanded(G3),as previously described by Marei et al.(2009). The nuclear maturation of oocytes was identified after aceto-orce in staining. Briefly,a total of 45oocytes were used in three independent replicates, allocating 5 oocytes per treatment(T-0, T-10 and T-100 ng/ml), and the cumulus cells were removed by gentle pipetting; denuded oocytes were fixed in fixation solution(acetic acid: ethanol; 1:3; v/v) at room temperature for 24 *h*. Then, the samples were put on a slide and covered with a square veneer of four drops of a paraffin-vaseline mixture (1:40).Next, the oocytes were stained with 1% aceto-orcein for 2 minutes, followed by rinsing with acetic acid,glycerol and water (1:1:3).The morphology of nucleus was observed under a stereomicroscope (LABOMED, USA).The extrusion of polar body 1(PB1) was examined under a stereomicroscope and considered as an indication of oocyte nuclear maturation for data analysis) Ni et al., 2015). Three replications were performed.

Sperm processing and *in vitro* fertilization (IVF)

Fresh semen from a ram of proven fertility was used for IVF. The motility was assessed using an inverted microscope and the motile spermatozoa were selected using the swim-up method as described by Di Francesco et al. (2011). At the end of IVM, the COCs (*n*=250)were completely denuded rom granulosa cells by gentle pipetting in H-TCM 199 consisting of 1mg/mL of hyaluronidase.Then,approximately15oocyteswereculturedin50 μL fertilization medium (Fert-TALP), containing 90mMNaCl, 25mM $NAHCO_3$, 12mM KCL,10mM sodium lactate,05mM $MgSO_4$, 0.5mM NaH_2PO_4, sodium pyruvate (0.018g/100mL), $CaCL_2$(0.147g/100mL), 3 mg/mL BSA (fatty acid free) and 50μg/mL gentamicin; 1×10^6 spermatozoa/mLwas added to the fertilization droplet. The gametes were co-incubated for 18 *h*at 38.5°C under 5% CO_2 in humidified air (Davachi et al., 2018).

In vitro culture

Eighteen hours after IVF, approximately 20presumptive zygotes per treatments were rinsed three times in culture medium and transferred to 50μL of synthetic oviductal fluid (SOF) droplets complemented with 2% basal medium Eagle's essential amino acids, 1% minimum essential medium nonessential amino acids, 1mmol/L glutamine, and 6 mg/mL bovine serum albumin (BSA) in a dish, coated with paraffin oil and incubated till day 8(day 0: IVF) at 38.5°C in a humidified atmosphere of 5% CO_2.The culture medium was renewed every 48 *h*. Cleavage was determined after 48*h*of cultivation, and the percentages of morula and blastocyst were estimated on days 4 and 8 (day 0 = IVF), respectively.Five replications were performed.

Quantitative reverse transcription PCR

The relative expression levels of *BAX a*nd *BCL-2* in blastocysts were assessed using quantitative reverse transcription PCR. Total RNA was extracted from pools of blastocysts(a total of 45 blastocysts were used in three independent replicates, allocating 5blastocysts per treatment)in each group utilizing Trizol reagent (Invitrogen, USA) and dissolved in 20 μl of RNase free water. The concentration and quality of the RNA were assessed by spectrophotometer (NanoDrop, USA). Briefly, DNase treated RNA was converted to cDNA using solvents and Takara guidance. The RT-qPCR solution was achieved in 20μL reaction with SYBR Green Mix (Takara, Japan) by Rotor-Gene 6000 RT-PCR (Corbett Research, Sydney, Australia). Primer sequences and average sizes of the amplified fragments are available in Table 1. The melt curve test was conducted during each run to test the existence of non-specific PCR products and primer dimers. Standard efficiency curves for each primer pair were calculated using a five 10-fold dilution sequence of positive control cDNA as a reference. The efficacy of the assays (E) was ≥95% and the standard curve R2 was ≥0.999. The relative expression levels of *BAX* and *BCL-2*were normalized to the endogenous normalizer (GAPDH), and $2^{-\Delta\Delta Ct}$ formula was applied for relative quantification (Livak andSchmittgen, 2001)

Table 1. Details of the primary sequences, the gene bank accession numbers, and the expected product size of the genes used for quantitative RT-PCR.

Gene	Sequence 5′→3′	Annealing Temperature (°C)	Amplicon Length (bp)	Accession No.
BAX	F:5-TGCAGAGGATGATCGCAGCTGTG-3′ R:5′-CCAATGTCCAGCCCATGATGGTC-3′	60	198	NM_173894
BCL-2	F:5′- ATGTGTTGGAGAGCGTCA-3′ R:5′-AGAGACAGCCAGGAGAAATC-3′	60	182	NM_001166486.1
GAPDH	F: 5′-CCTGAGACAAGATGGTGAAGGT-3′ R: 5′-ATGGGTGGAATCATACTGGAAC-3′	60	164	NM_001190390

F: forward; R: reverse; apoptotic *BAX*, anti-apoptotic *BCL-2,* and *GAPDH:* glyceraldehyde-3-phosphate dehydrogenase

Table 2. Effect of different concentration of Triiodothyronine on cumulus expansion in ovine COCs (three replicates)

Groups	Total COCs	%(N) of oocytes with cumulus cells		
		G1 %(n)	G2 %(n)	G3 %(n)
T-0 ng/mL	150	72%(108)[a]	21.3%(32)[a]	6.67%(10)[a]
T-10ng/mL	150	65.33% (98)[a]	16.67%(25)[a]	18%(27)[b]
T-100ng/mL	150	36%(54)[b]	34%(51)[b]	30%(45)[c]
df		2	2	2
Chi-Square		45.38	12.99	29.12
P-value		<.0001	0.0015	<.0001

[a, b, c] Different superscripts within the same column indicate a significant difference (P<0.001). T: Triiodothyronine, n; number; G1: Full expansion, G2: Partial expansion and G3: Without expansion (logistic regression; The GENMOD Procedure followed by chi-square test).

Table 3. Effect of Triiodothyronine during IVM on ovine oocyte maturation, cleavage and blastocyst rate.

Treatment	Total Oocytes	Cleavage, (n)		Morula, (n)		Blastocysts, (n)	
T-0	149	88.64±6.02[a]	(132)	60.41±5.35[a]	(90)	40.92±8.50[a]	(61)
T-10	145	87.59±3.93[a]	(127)	68.28±5.66[b]	(99)	42.07±7.48[a]	(61)
T-100	142	48.69±7.54[b]	(69)	31.67±5.41[c]	(45)	22.51±3.81[b]	(32)
P-value		*P*<0.001		*P*<0.001		*P*<0.01	

Data are presented as absolute values (mean of percentage±standard deviation) (five replicates).[a,b,c]Different superscripts letters indicate significant differences among treated groups. One-way ANOVA and Duncan's multiple range tests for post hoc were used to test the differences between the treatments.

Statistical analysis

Normally distributed variables were tested using student´s t-test. If data could not be normalized on log transformation, the Mann-Whitney test was used. Logistic regression modelsusing the GLIMMIXprocedure followed by Chi-Square and one-way ANOVA tests were used for categorical variables (SAS version 9.2, Inc., Cary, NC, USA).Significance was set at 0.05 in all cases (P<0.05).

RESULTS

Effects of exogenous T3 on IVM

The highest full cumulus expansion in COCs at the end of IVM was observed in the control group (T-0) (Table2). No significant differences were noticed between the 10*ng/mL*T3 and the control group (Table2). However, the highest concentration (100*ng/mL*) of T3 significantly reduced the proportion of COCs with the fully expanded cumulus cells compared to T-0 and T-10 groups (P<0.001).At the end of IVM, the percentage of oocyte in the metaphase II (MII,based on extrusion of polar body 1)was higher inthe control (62.41 %) compared to 100*ng/mL* of T3 group(41.54%, P<0.0004). The addition of 100*ng/mL* T3 to the maturation medium substantially decreased (P<0.0004) the rate of nuclear maturation to MII compared to the addition of 10*ng/mL* of T3(41.54% vs 67.58 %, respectively, P<0.0004). Nevertheless, the rate of nuclear maturation of oocytes under the IVM medium with 10*ng/mL*T3 was not significantly different from the control group (67.58%vs.62.41, respectively).

Effects of exogenous T3 on blastocyst development and quality

The results of cleavage and blastocyst formation are summarized inTable 3. Our findings showed that the addition of the high concentration (100*ng/mL*) of T3 to IVM medium dramatically decreased the cleavage rate compared with that of theT-10and control groups. The proportion of cleaved embryos developed to the morula stage at day 4 (day 0 - IVF) in the T-100*ng/*mL group was lower ($P< 0.001$) than the control and T-10*ng/mL* groups. The highest rate of blastocyst formation at day 8was recorded in the T-10*ng/mL*group. However, no statistically significant differences in the mean cleavage and blastocyst formation rate were observed between the T-10*ng/mL*and control embryos (Table3).

Effect of exogenous T3 on apoptotic genes expression in blastocysts

A significantly higher expression level of *BAX* ($P<0.001$) and a dramatically lower expression level of *BCL2* ($P< 0.01$) were recorded in the T-100*ng/mL* group compared to the control group (Figure1). Furthermore, the relative mRNA levels of *BCL2* and *BAX* were significantly higher in the T-10*ng/mL* group compared to the control ($P<0.01$, Figure1).In addition, the ratio of the *BAX: BCL2*expression in blastocysts was significantly decreased in the T-10 compared with the T-100ng/mL group (Figure 1).

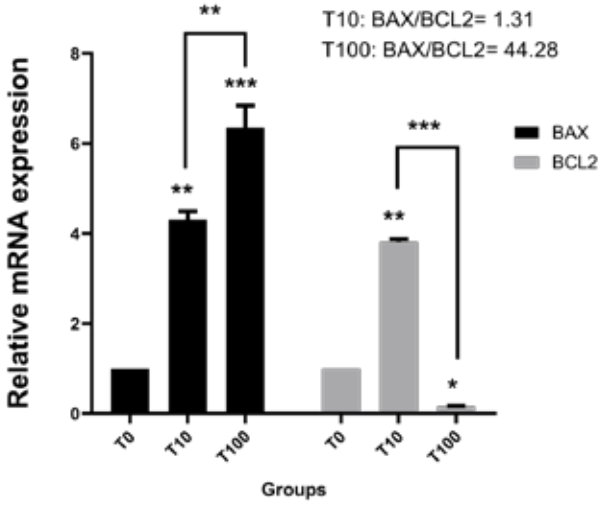

Figure 1. Effect of different concentration of Triiodothyronine (T3) on the relative mRNA expression of *BCL2* and *BAX* in ovine blastocysts. T: Triiodothyronine. Bars with star mark (*) represent groups that were different from control and between treatments group (*$P<0.05$, ** $P<0.001$;***$P<0.0001$).

DISCUSSION

Several studies reported that THs might influence ovarian metabolism and embryogenesis on mouse (Cecconi et al., 1999) and bovine (Costa et al., 2013). In this research, the effects of T3 supplementation to the IVM media on the meiosis process, expansion rate of cumulus cells, and blastocyst formation rate of ovine oocytes were investigated. Our results indicate that the treatment of ovine oocytes with 100 *ng/mL* of T3 during IVM decreases the nuclear maturation rate. The results were in line with the study of Ashkar et al. (2010b), which have noted that high concentrations of THs during bovine oocyte maturation reduced progression from MI to MII. However, when IVM media were supplemented with low concentrations of THs the percentage of MII stage oocytes was not different from that of controls.It seems that there is a cut-off point above which the effects of T3 are detrimental for embryo development.

The current research indicates that the addition of 10 *ng/mL*T3 to the IVM medium results in higher oocyte maturation, cleavage, and blastocyst rate when compared with 100*ng/mL* T3. Consistent with our results, Costa et al. (2013) revealed that the addition of 50ng/mL of T3tothe maturation medium could promote the nuclear maturation and fertilization rate of bovine oocytes.It has been indicated that T3 might foster oocyte maturation by transcribing and accumulating certain mRNA molecules (Regassa et al.,2011). Therefore, TH scan promote transcription after the maturation phase and may enhance the efficacy and quality of the rainbow trout embryonic development(Raine et al.,2004).

In contrast, Cecconi et al.(1999)stated that supplementation with THs had no beneficial effects on murine embryonic development. THs treatment could probably impose an adverse effect on follicle cells or oocyte metabolism by a decline in aromatase activity, inability of follicles to progress through the antral stage, or delayed meiotic process following GVBD(Cecconi et al.,1999; Cecconi et al., 2004).Although these results may be species-specific, dose-dependent effects of THs supplementation on blastocyst formation and hatching rate of bovine oocytes have been reported (Ashkar et al., 2010b)as well as in our study.

Ashkar et al. (2010b) reported that the supplementation of IVM medium with different concentrations of T3 or a combination of T3 and T4 in bovine did not influence cleavage and blastocyst rates.In the present study, there was no significant difference between the

supplementation of ovine oocytes maturation medium with low-dose (10 ng/ml) of T3 and the control group. Although the low-dose (10 *ng/mL*) supplementation improved blastocyst formation rate, this improvement was not significant. In line with our results,Ashkar et al.(2010a) indicated that T3 supplementation at the dose of 50 *ng/mL*enhanced the blastocyst formation rate in dairy cattle.It has been demonstrated that the apparent improvement in embryonic development might be in part related to up-regulatory effects of THs on certain responsive genes involved in cellular proliferation, improvement in mitochondrial function and adequate production of energy (Ashkar et al.,2010b;Cecconi et al.,2004). However, we observed that the addition of 100*ng/mL* of T3 during IVM was harmful to oocyte development and exerted an inhibitory effect on maturation, cleavage and blastocyst rate *in vitro*, indicating that the effects of maturation medium supplementation with T3 could be species-specific and dose-dependent.

In contrast with our result, Ashkaret al.(2010b) demonstrated that the addition of 100 *nM*of T3 to the maturation medium increased the rate of bovine blastocysts on the eighth day of culture. It has been shown that higher THs concentrations in IVM medium were more effective at inducing progesterone and and rostenedione production in cultured bovine follicle cells(Spicer et al.,2001).We agree with Ashkar et al., (2010b) who reported that it is difficult to make direct comparisons between studies, because these findings might be species-specific and the effects of THs might be dose dependent.

Apoptosis is a basic physiological mechanism and plays an important role in renewing the normal cells and eliminating the abnormal cells for the multicellular organism. *BCL2*family proteins are involved in the modulation of apoptosis and expected to combine signals from survival-inducing and death-promoting mechanisms(Burlacu,2003).It has been reported that, THs inhibit apoptosis and granulosa cells' death through mitochondrial pathways (Asahara et al.,2003). To elucidate the molecular mechanisms of THs mediated improvement in blastocyst quality, we analyzed the mRNA expression of *BAX* and *BCL2* genes in blastocyst stage of embryos.The results of the present study clearly showed that the addition of 10 ng/mL of T3 to maturation medium increased the expression of anti-apoptosis related genes (*BCL-2)* in the blastocysts.Feugang et al. (2011)indicated that anti-apoptotic genes (*BCL2-like1*) are important for the survival of embryos and *BCL2-like1*/*BAX* ratios were always in the direction of *BCL-2 like1* transcripts, which is favorable to embryonic survival. Our results revealed that the relative expression level of *BAX* was significantly higher in the T-100 *ng/mL*group compared to the T-10*ng/mL*.Moreover, a decrease in *BAX*/*BCL2*ratio was observed in the T-10 treatment compared to T-100*ng/mL*.It was also noticed that the expression of *BAX*was lower in the T-10 group than in the T-100*ng/mL* group, and the ratio of*BAX to BCL-2*transcripts was up-regulated in T-100*ng/mL* group. Rao et al., (2012) demonstrated that the ratio of *BAX* to *BCL2*might be used to measure the tendency of oocytes and embryos towards either survival or apoptosis,and it might also be associated with the ability of oocytes to complete nuclear maturation as reported by Filali et al.,(2009).In other words, the addition of high doses ofT3tothe IVM medium led to a significantly lower rate of blastocyst formation and promoted ovine blastocyst cell apoptosis.

CONCLUSION

In general, the addition of thyroid hormone to the IVM medium had controversial effects on the development of ovine oocytes. The lower dose of T3 (10 *ng/mL*) could reduce the rate of apoptosis in blastocysts; whereas the higher dose (100 *ng/mL*) imposed detrimental effects.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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