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Full Length Research Paper

Retinoic acid effects on nuclear maturation of bovine oocytes *in vitro*

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In the present study, the effect of all-trans retinoic acid (t-RA) administration during *in vitro* maturation (IVM) on bovine oocytes maturation was determined. Concentrations of t-RA (RA; 0, 0.25, 0.5 and 1 µM) and 0.1% ethanol (vehicle) were included in the maturation medium. Ovaries collected from the local abattoir were transported to the laboratory in in 0.9% NaCl with 100 IU/ml penicillin and 100 µg/ml streptomycin at 30 - 35°C within 1-2 h after collection. The oocytes of antral follicles, 2 to 8 mm in diameter, were recovered by aspiration. After preliminary evaluation, the oocytes were selected and washed four times in HEPES-TCM 199 supplemented with 2% FBS, 0.2 mM sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin. Then 10 cumulus-oocyte complexes (COCs) were subjected to each droplet of maturation medium and incubated at 38.5°C, 5% CO_2 and 95% humidity for 24 h. Maturation medium was bicarbonate-buffered TCM199 supplemented with 10% FBS, 0.2 mM sodium pyruvate, 5 µg/ml bovine FSH, 0.01 IU/ml bovine LH, 100 IU/ml penicillin and 100 µg/ml streptomycin. Results show different concentrations of t-RA have no effect on cumulus expansion. The rate of oocytes developing to the MII stage compared to control, vehicle, and 0.25 µM groups was significantly increased with 1 µM t-RA treatment ($p<0.05$). In conclusion, 1 µM retinoic acid significantly increased the bovine oocyte maturation in the commercial TCM199 medium.

Key words: *in vitro* maturation, retinoic acid, bovine oocytes.

INTRODUCTION

Mammalian oocytes arrested at the diplotene stage of first meiotic prophase spontaneously resume meiotic maturation once released from their follicular environment. Compared with *in vivo* maturation, *in vitro* maturation (IVM) conditions are simple and materially limited, which can profoundly affect the maturation status of oocyte. For this reason, improved culture conditions are essential to obtain consistently successful and relia-ble oocyte maturation (both cytoplasmic and nuclear), leading to marked improvements in the efficiency of *in vitro* embryo production (Alminana et al., 2008). Improve-ment of bovine oocytes IVM has been achieved mainly by the introduction of gonadotropins (Younis et al., 1989; Alves et al., 2001), estradiol (Brackett et al., 1989), vita-mins (Bortolotto, 2000; Duque et al., 2002b), and several growth factors into the media (Bortolotto, 2000).

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Vitamin A is one of the micronutrients which have been implicated in cattle reproduction (Gomez et al., 2004). Early research showed the positive effects of vitamin A supplementation on bovine fertility in artificial insemination, and several studies on effects of vitamin A metabolites used in other assisted reproductive techniques (ART), including superovulation, ovum pick up, and *in vitro* maturation culture have provided evidence for the specific roles of vitamin A in oocyte cytoplasmic maturation (Ikeda et al., 2005). The beneficial effect of vitamin A during oocyte growth *in vivo* has been reproduced by retinol derivatives added to an *in vitro* culture system in which the oocytes are meiotically arrested (Duque et al., 2002). Recently it has been shown that transcription factor, increases midkine (MK) expression in bovine cumulus-granulosa cells in response to RA (Gomez et al., 2003). MK is one of the heparin-binding growth differentiation factors that are known to be quite rich in bovine follicular fluid (Ohyama et al., 1994). In addition, RA seems to have a role in preventing oxidative stress in the cell (Ahlemeyer et al., 2001). However, most of the

available information about retinoids is related to advanced stages of embryonic development, and little is known about the RA-dependent gene expression and posttranscriptional modifications in the oocyte (Gomez et al., 2004).

In the live cell, all-trans-RA binds to retinoic acid recaptors (RAR; α, β, and γ) and its isomer 9-cis of retinoic acid (9-cis-RA) specifically binds to retinoid X receptors (RXR; α, β, and γ) (Mangelsdorf et al., 1994; Chambon, 1996). However, under *in vitro* conditions, 9-cis-RA activates both RXR and RAR. The beneficial effects RA have been demonstrated in bovine oocytes, especially on cytoplasmic competence after IVM (Duque et al., 2002; Gomez et al., 2003) and embryonic development and quality (Duque et al., 2002; Gomez et al., 2003; Hidalgo et al., 2003; Livingston et al., 2004; Lima et al., 2006). Despite the beneficial effects of retinol, an applicable dose for the different retinol metabolites is yet to be determined.

This study was designed to evaluate the effects of supplementation of the IVM medium with different concentrations of the t-RA on nuclear maturation of bovine oocytes.

MATERIALS AND METHODS

All chemicals were purchased from Sigma, unless otherwise indicated.

Collection of cumulus-oocyte complexes (COCs)

Bovine ovaries (n = 157) were collected at a local slaughterhouse in a termoflask and were transported to the laboratory within 1 h after collection in 0.9% NaCl containing 100 IU/ml penicillin and 100 µg/ml streptomycin (15140-122; Gibco/Invitrogen) at 30-35°C. Ovaries were washed twice in freshly prepared saline without antibiotic. Visible follicles 2 to 8 mm in size were aspirated through an 18-gauge needle connected to a 10 cc syringe, and the contents recovered into a 50 ml conical tube and allowed to settle for 10 min, then COCs were rinsed four times with Oocyte Collection Medium (OCM), which consisted of HEPES-TCM 199 (M-2520) supplemented with 2% FBS (10270-106; Gibco/Invitrogen), 0.2 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 IU/ml of heparin (drug store).

In vitro maturation

Only oocytes enclosed in a compact cumulus with evenly granulated cytoplasm for maturation were selected. The COCs were washed four times in OCM and one time in oocyte maturation medium (OMM), OMM was bicarbonate-buffered TCM199 (31100-027; Gibco) supplemented with 10% FBS, 0.2 mM sodium pyruvate, 5 µg/ml bovine follicle stimulating hormone (bFSH) (Sioux Biochemicals, Sioux Center, IA), 0.01 IU/ml bovine luteinizing hormone (bLH) (Sioux Biochemicals) and 100 IU/ml penicillin and 100 µg/ml streptomycin. Oocyte maturation was performed under mineral oil by culturing approximately 10 COCs in 50 µl of maturation medium in 60 × 15 dishes at 38.5°C in 5% CO_2 in air and high humidity for 24 h. For its use in IVM, all-trans-RA was solved in ethanol, aliquoted, and stored at - 20°C in the darkness.

Assessment of the cumulus expansion and nuclear status

COCs which showed fully expanded cumulus cells after 24 h maturation period, were assessed and those whose COCs did not expand or showed incomplete expansion were not taken into account.

Oocytes were then placed in a 1% sodium citrate solution for 15 min at room temperature (Tarkowski, 1966), followed by pipetting to remove the cumulus cells. Denuded oocytes were recovered and transferred to glass slides. Vaseline and paraffin wax were used to keep the coverslip in contact with the oocytes. The slides were immersed in ethanol:acetic acid (3:1) for at least 24 h to fix them (Sun et al., 2001). Oocytes were then stained with 1% (w/v) aceto-orcein, and to decolorize, aceto-glycerol (20, 20 and 60%; glycerol, acetic acid and distillated water, respectively) was used (Hewitt et al., 1998) and examined under a phase-contrast microscope at 400× magnification. Oocytes were classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) stage of the maturation process (Kubelka et al., 1988).

Experimental design and statistical analysis

In the current study different concentrations of t-RA (RA; 0, 0.25, 0.5 and 1 µM) and 0.1% ethanol (vehicle) were included in the maturation medium. One-way ANOVA was used to determine significant differences in data levels. Tukey test was followed to determine statistical differences between groups. Statistical analyses were preformed using PROC GLM of SAS software (Version 9.1). The significant differences among the treatments were determined when $p < 0.05$. Data are expressed as mean ± S.E

RESULTS

In this experiment 735 immature bovine oocytes were selected and randomly allocated to control, vehicle and treatments. After 24 h culture, there was no significant difference in cumulus expansion between control and vehicle within treatments and also among treatments (Table 1).

After assessment of cumulus cells expansion, 602 oocytes were stained and nuclear status was evaluated. The percentage of the oocytes remained at the GV stage was 0 in whole groups. There was no significant difference among groups at GVBD and MI stage. There was no significant difference in MII stage among control, vehicle, 0.25, 0.5 µM t-RA. The rate of oocytes developing to the MII stage of maturation compared to control, vehicle, and 0.25 µM t-RA with 1 µM t-RA was significantly increased ($p<0.05$). But there was no significant difference between 0.5 and 1 µM t-RA in MII stage (Table 2).

DISCUSSION

Retinol metabolites have been recommended as important components of IVM media to improve cytoplasmic maturation and embryo development. Beneficial and detrimental effects of the retinol metabolites have been described for bovine embryo development *in vitro* (Duque et al., 2002; Gomez et al., 2003; Hidalgo et al., 2003; Lima

Table 1. Effect of t-RA on cumulus expansion of bovine oocyte.

t-RA concentrations (µM)	Cultured oocytes (n)	Cumulus expansion rate, n (%)
0 (Control)	144	117 (81.07±1.10)[a]
Ethanol 0.1% (vehicle)	150	123 (82.40±1.10)[a]
0.25	148	122 (82.42±1.10)[a]
0.5	143	120 (84.11±1.10)[a]
1	150	125 (83.31±1.10)[a]

Data are expressed as mean ± S.E.
[a]Different superscripts within the same column represent significant differences ($P < 0.05$).

Table 2. Nuclear status of bovine oocyte cultured in different concentration of t-RA.

t-RA Concentrations (µM)	Assessed oocytes (n)	Nuclear status			
		GV, n (%)	GVBD, n (%)	MI, n (%)	MII, n (%)
0 (Control)	119	0	20 (16.19±2.09)[a]	19 (15.76±2.37)[a]	80 (67.21±1.47)[a]
Ethanol 0.1% (vehicle)	111	0	16 (14.27±2.09)[a]	19 (18.33±2.37)[a]	76 (67.39±1.47)[a]
0.25	122	0	20 (16.28±2.09)[a]	18 (16.02±2.37)[a]	84 (68.65±1.47)[a]
0.5	120	0	14 (11.98±2.09)[a]	20 (16.36±2.37)[a]	86 (71.65±1.47)[ab]
1	130	0	15 (9.96±2.09)[a]	17 (13.86±2.37)[a]	99 (75.12±1.47)[b]

Data are expressed as mean ± S.E.
[a,b]Different superscripts within the same column represent significant differences ($P < 0.05$). GV = germinal vesicle; GVBD = germinal vesicle breakdown; MI = metaphase I; MII = metaphase II.

et al., 2006). To the best of our knowledge, there are no reports on the effects of t-RA on the nuclear maturation of bovine oocytes.

The effects of retinol (RT) and RA on oocyte maturation are not yet totally explained in IVP systems, but both, directly or indirectly, can improve nuclear (Bortolotto, 2000) and cytoplasmic competence (Duque et al., 2002a). It is believed that cumulus cells are stimulated by gonadotrophin (FSH) and growth factor (EGF) to produce and secrete Hyaluronic acid, which results in the expansion process (Gordon, 2003). Certain constituents of the maturation medium may influence cumulus cell expansion, glutamine, which is a constituent of TCM-199, being an example. Also, serum in maturation medium improved oocyte maturation (Gordon, 2003). In the current study, oocytes were matured in OMM supplemented with FSH, LH and FBS. But, we were not able to find significance effect of t-RA on COCs expansion. So, probably the presence of these components during maturation may have masked the effects of t-RA on cumulus cell expansion of bovine oocytes.

In this study, we examined the effects of a range of t-RA concentrations during IVM. Compared to the control group, nuclear maturation rates in 1 µM RA was significantly improved. Alminana et al. (2008) suggested that highest concentrations of 9-cis RA and all-trans retinol metabolites (500 nm RA and 12500 nm ROH) were cytotoxic to oocytes nuclear maturation. The different concentrations of retinoids used among studies could also have been the reason for these discrepancies, given that the effects of these compounds are dependent on their concentration (Gomez et al., 2003; Livingston et al., 2004; Lima et al., 2006).

A possible explanation for these discrepancies could be the different IVM conditions used by others, especially varied hormone supplementation regimens. Under IVM conditions, unphysiologically high concentrations of gonadotrophins may induce the aberrant acquisition of FSH-R and LH-R, although the FSH and LH dependency of preovulatory follicular cells seems to be profoundly regulated *in vivo* maturation (Webb et al., 2003). It is possible that RA attenuates aberrant differentiation of cumulus-granulosa cells, thereby preventing oocytes from premature and abnormal maturation (Moor et al., 1985; Assey et al., 1994).

Recently, t-RA has been shown to increase MK mRNA at concentrations of 0.1 – 0.3 µM in rat cultured granulosa cells (Minegishi et al., 1996). It is also found that MK suppressed apoptosis in the cumulus cells during the IVM period of bovine COCs (unpublished data). Although we did not assay the effect of RA on MK expression, but it is possible that RA enhances oocyte nuclear maturation through the production of MK in cumulus cells in the *in vivo* and/or *in vitro* maturation of COCs.

In animal cells, the majority of mRNAs have a 3'poly-(A) tail. During oocyte growth, large amounts of mRNA are synthesized and polyadenylated in the nucleus. Poly-(A) tails have been shown to regulate both translation and mRNA stability. Requirements for IVM include polyadenylation of mRNAs coding proteins for chromatin con-

densation, spindle assembly, and activation of MPF and MAP kinase (Krischek and Meinecke, 2002). In response to RA, the higher polyadenylation observed, suggests increased mRNA quality as well as increased processing (Gomez et al., 2004). Oocyte in *in vitro* conditions are under oxidative pressure and their defensive mechanism are not effective; so, to protect oocytes against these oxidative components, anti-oxidants should be added to their medium. Previous studies have shown that retinoids participate in a biological anti-oxidant network and have been implicated as important regulators of redox signalling pathways (Olson, 1993; Imam et al., 2001; Ikeda et al., 2005). Furthermore, it has been reported that retinoids protect against oxidative damage by maintaining adequate endogenous competency and levels of anti-oxidants that are essential for oocyte maturation, fertilization and embryonic development (Guerin et al., 2001). We suggest that in our study, some useful effects of included RA partly have been due to this protection effects RA in oocyte maturation.

In conclusion, the present study showed that t-RA, at a concentration of 1 µM, in the IVM medium significantly increased oocyte maturation. Additional studies are needed to elucidate the possible mechanisms by which t-RA influences the nuclear maturation of bovine oocytes.

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