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Effect of Isotretinoin on Nuclear Maturation and Fertilization Rate of Bovine Oocyte

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ABSTRACT

The blastulation rate following in vitro maturation, fertilization and culture of bovine oocytes is generally low. Numerous studies have reported that oxidative stress (OS) has detrimental effects on quality of gametes and embryos under culture conditions. Thus, adding some substances to IVM and IVF media could protect oocytes and early embryos against oxidative stress.

The purpose of this study was to evaluate whether enriching the oocyte in in vitro maturation medium with isotretinoin (13-cis-retinoic acid) would improve the in vitro embryo production efficiency in cattle. To this end, 250 oocytes were collected and divided into five groups of 50 oocytes: Group 1: Washing medium (WM) and maturation medium (MM) without retinoic acid; group 2: The WM supplemented with 5 nm isotretinoin and the MM without retinoic acid; group 3: The WM without retinoic acid and the MM plus 5 nm isotretinoin; group 4: The WM supplemented with 5 nm isotretinoin and the MM plus 5 nm isotretinoin; and group 5: The WM without retinoic acid and the MM plus 10 nm isotretinoin.

The results showed that nuclear maturation in the groups 3 and 4 had higher percentage than that in the groups 1, 2 and 5. The percentage of oocytes reaching the metaphase II stage significantly increased (P<0.05) in the 5 nm RA-treated groups compared to groups without retinoic acid (58.30%) and 10 nm RA (50%). RA supplementation significantly improved the fertilization rate (76%) compared with groups without retinoic acid (56%). The results demonstrated that addition of 5 nm isotretinoin to the in vitro oocyte maturation medium enhanced the maturation and fertilization rates of bovine oocyte.

Key words: Bovine, Oocyte, *In vitro* maturation, Isotretinoin

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INTRODUCTION

Oxidative stress (OS) has detrimental effects on quality of gametes and embryos under culture conditions [1]. Free radicals such as reactive oxygen species (ROS) interact with oocytes and embryos, and decrease blastulation rates in embryo development [2].Most mammalian cells possess efficient antioxidant systems such as catalase or superoxide dismutase, as well as thiol compounds that act as metabolic buffers scavenging active oxygen species [3]. These systems are more critical for important processes like maturation, creation and growth of gametes and embryos.

The procedures for *in vitro* production (IVP) of embryos consist of *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) of embryos. The IVP is a sequential process and failure in each of its steps could influence the final result [4]. Progress toward simplification and modification of a medium for the IVP, especially for reduction of oxidative stress, could increase the efficacy of embryo production in commercial aspects. In recent years, various materials with antioxidative properties have been examined in media of the IVP.

Vitamin A is one of the fat-soluble unsaturated isoprenoids, and is well known to regulate development, cellular growth and differentiation, and maintenance of tissue function [5,6].

The requirement for vitamin A in mammalian reproduction was first recognized a century ago. Nowadays, studies show that all-*Trans* retinoic acid (RA)

has crucial effects on male and female reproduction function as well as on embryonic development [7].

Vitamin A could influence bovine ovarian follicular development [8], steroidogenesis [9], uterine environments, oocyte maturation, and embryo and conceptus development [10]. Vitamin A along with its metabolites on bovine reproduction not only is regarded as an antioxidant, but also affects as a local modulation on development and differentiation of cells. Xing et al. showed that retinoids had direct effects on modulation of the gonadotropin receptor promoter [11].

Vitamin A can also affect reproduction through induction of midkine by the RA in cumulus-granulosa cells [12]. MK is a nonglycosylated protein, composed of two domains held by disulfide bridges. It is a developmentally important retinoic acid-responsive gene product strongly induced during mid-gestation, hence the name midkine. The concentration of midkine is high in bovine follicular fluid [13], and it can suppress apoptosis in cumulus cells. It is possible that the RA induces production of midkine (MK) in cumulus cells; the MK promotes the maturation of bovine oocytes as well as subsequent fertilization and development to blastocysts [14].

Some studies have suggested that retinoic acid may regulate the nitric oxide synthesis in granulosa cells [15]. Nitric oxide is a free radical with physiological roles, which acts as a cell-signaling molecule modulating a variety of molecular pathways in various tissues such as reproductive organs. Santana et al. showed that enhancement of nitric oxide production in bovine embryo culture medium may help development of preimplantation bovine embryos [16].

Vitamin A is a term reserved to designate any compound consisting of four isoprenoid units with five carbon-carbon double bonds, which possesses the biological activity of retinol. There are many kinds of metabolites for retinol in different cells and tissues. Some common forms of retinol are 9-*cis*-retinal forming photoactive isorhodopsin, 11-*cis* retinal present in the retina and isotretinoin as metabolites of retinol found in many tissues [17].

Since the two last decades, a number of studies have examined different concentration of 9-*cis*-retinal on the efficacy of bovine IVP media.

Results of some studies carried out on the issue in Spain showed that addition of 9-*cis*-retinal into the IVM medium increased the bovine blastocyst development and hatching rate [18]. Duque et al. showed that the cytoplasmic competence of bovine oocytes improved in the presence of 9-*cis*-retinal during pre-maturation [19]. Livingston et al. demonstrated that addition of retinol to the IVM medium may improve the embryonic development and blastocyst rate of bovine oocytes [20].

The aim of this study was to verify whether increasing the availability in the washing medium (WM) and maturation medium (MM) of isotretinoin would improve the overall *in vitro* embryo production (IVEP) efficiency in cattle. The study evaluated the effects of supplementation of the WM and IVM medium with isotretinoin compounds on nuclear maturation rate and pronuclear formation following fertilization and cleavage rate.

MATERIALS AND METHODS

A number of 250 oocytes were collected and divided into five groups of 50 oocytes: group 1=the WM without retinoic acid and the MM without retinoic acid; group 2=the WM+5 nm isotretinoin and the MM without retinoic acid; group 3=the WM without retinoic acid and the MM+5 nm isotretinoin; group 4=the WM + 5 nm isotretinoin and the MM+5 nm isotretinoin; and group 5=the WM without retinoic acid and the MM+10 nm isotretinoin. The concentration of the RA was based on previous findings indicating that the developmental competence of bovine oocytes was enhanced with 5 nm 9-*cis* RA. In each group, to assess maturation, 12 oocytes were stained with Hoechst and 13 oocytes were morphologically examined with a microscope. The 25 oocytes mature were evaluated in terms of fertilization rate. For the purpose of experiment, three trials were undertaken. For each trial, data were pooled from two culture plates.

Oocyte recovery

Cow ovaries were collected from a slaughterhouse and placed in a thermoflask containing 0.9% NaCl with 100 U penicillin/ml and 100 µg streptomycin/ml at 30°C-35°C. The ovaries were transported to the reproductive biotechnology laboratory in less than 1 hour. Then, the ovaries were washed twice in distilled water and once in freshly prepared saline. COCs were aspirated from 2-8 mm visible follicles with the aid of an 18-gauge needle attached to a 10 ml sterile disposable syringe and recovered into a 50 ml corning tube. Follicular fluid and the COCs were placed in an ovum concentrator (Em-Con, Comextrade, Tarragona) and the COCs were washed three times in a washing medium (90% H-TCM199, 10%FCS).

In vitro maturation (IVM)

Only oocytes enclosed in a compact cumulus with evenly granulated cytoplasm were selected for maturation. The COCs were incubated for 22-24 h (using 5-10 COCs per 50-100 µl of maturation medium) in tissue culture dishes (35 mm × 10 mm, Nunclon 153066, Inter-med., Roskilde, Denmark) under mineral oil in a humidified incubator

(Forma Scientific 3111 Series, Forma Scientific Inc., Marietta, OH, USA) gassed with 5% CO_2 in air at 38.5°C. The base of IVM medium was 25 mM HEPES-buffered TCM199 supplemented with 2 mM sodium pyruvate, 1 mM l-glutamine, gentamicin (50 µg/ml), 10% steer serum, porcine FSH (1 µg/ml), and 17b-estradiol (1 µg/ml).

Assessment of nuclear maturation status and pronuclear formation

The oocysts in each of the groups were examined in terms of morphology with a microscope. Afterwards, the oocysts as stripped of cumulus cells were evaluated in the working medium containing hyaluronidase (0.01% w/v) under mineral oil for the presence of a polar body under the stereomicroscope. Subsequently, the oocytes were fixed in 4% formaldehyde and stained with Hoechst 33342 (Sigma-Aldrich) for their nuclear maturation status.

In vitro fertilization (IVF) and *in vitro* culture (IVC)

Sperm separation was carried out using a swim up procedure similar to that reported by Parrish et al. [21]. Briefly, semen from one frozen straw of a single bull was thawed in a water bath and added to a polystyrene tube containing 1 ml of pre-equilibrated Sperm-Tyrode's albumin lactate pyruvate (TALP). After 1 hour of incubation, approximately 700 µl of the upper layer of the supernatant containing the motile spermatozoa was removed. The spermatozoa were then centrifuged for 7 min at 200 g, and the supernatant was aspirated to leave a pellet of approximately 100 µl. The concentration of the spermatozoa was determined with a haemocytometer. Moreover, the IVF was accomplished by incubating the oocytes and sperm cells together in fertilization medium (Modified Tyrode's solution , supplemented with 0.6% fatty acid-free BSA, 0.2 mM sodium pyruvate, 2 µg/mL heparin, and 50 µg/mL gentamicin) for 18–20 h at 39°C in 5% CO_2 and high humidity [21]. The presumptive zygotes were removed from the IVF droplets, denuded of cumulus cells by repeated pipetting, washed several times in a pre-warmed culture medium, and finally transferred into IVC droplets made of modified synthetic oviductal fluid (mSOF) added to 1 mM glucose and 3 mg/ml bovine serum albumin (BSA). The embryo culture was maintained at 39°C, 5% CO_2, 5% O_2 and 90% N_2 under mineral oil and examined under a stereomicroscope after 48-72 hours for evaluate the fertilization rate.

Statistical analysis

Each experiment was repeated at least three times. Data were compared by X2 tests using the statistical analysis software SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Differences of $P<0.05$ were considered significant.

RESULTS

A total of 250 oocytes aspirated from the ovaries were matured *in vitro* and evaluated for cumulus expansion,

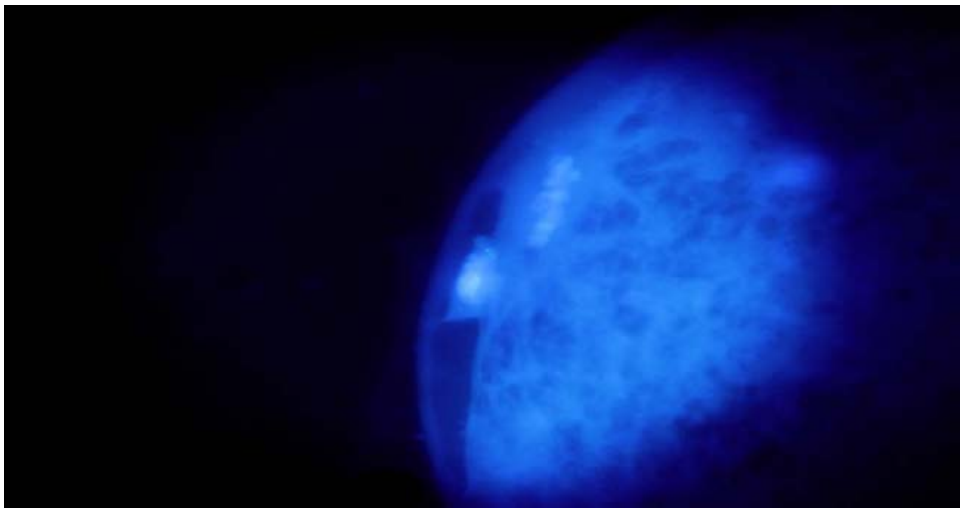

Figure 1: Oocytes stained with Hoechst (nuclear maturation)

Table 1: Effect of the 13 *cis* retinoic acid (RA) on the cumulus expansion, nuclear maturation and fertilization rate in the bovine oocytes

Group	Number of oocytes	Number of replicates	Cumulus expansion(%)/13 oocyte	Nuclear maturation (MII)%/12 oocyte	Fertilization (%)
1	50	3	69.2	58.30a	56.00a
2	50	3	61.5	66.70ab	60.00a
3	50	3	69.2	91.70c	76.00b
4	50	3	84.6	83.30bc	76.00b
5	50	3	61.5	50.00a	52.00a

Different superscripts in the same column differ significantly: a,b,c; $P<0.05$.

nuclear maturation (MII) and fertilization rate. The experiments were repeated three times. Results are shown in Table 1. The oocytes used for analysis of the cumulus expansion were also used for analyzing the fertilization rate.

In the present study, cumulus expansion in group WM 5 nm RA+MM 5 nm RA showed a higher percentage than other groups but this difference was not significant.

The results of this study showed that nuclear maturation in groups 3 and 4 was the higher percentage than groups 1, 2 and 5. The percentage of oocytes reaching the metaphase II stage was significantly increased ($P<0.05$) in the 5 nm RA-treated groups in MM compared with without retinoic acid (58.30%) and 10 nm RA (50%). RA supplementation in MM significantly improved fertilization rate (76%) compared with without retinoic acid (56%).

In the present study, nuclear maturation (Figure 1) in groups WM 0 nm RA+MM 5 nm RA and WM 5 nm RA+MM 5 nm RA had a statistically significant difference with Group WM 0 nm RA+MM 0 nm RA, Also It was shown groups that were added in maturation medium dose of 5 nM RA nuclear maturation rates were higher than the group that 10 nM RA was added to the maturation medium. The latter group showed the lowest maturation rate compared to the other groups.

A significant difference was observed in nuclear maturation between Groups WM 0 nm RA+MM 5 nm RA and WM 5 nm RA+MM 5 nm RA to group wm 0 nm RA+mm 10 nm RA.

In this experiment 25 mature oocytes from each group transferred to fertilization medium and the rate of fertility was assessed, in groups WM 0 nm RA+MM 5 nm RA and WM 5 nm RA+MM 5 nm RA showed the highest percentage (76%) and lowest percentage in group wm 0 nmRA+mm 10 nm RA (52%). A significant difference in fertility rates between groups WM 0 nm RA+MM 5 nm RA and WM 5 nm RA+MM 5 nm RA with the other groups was observed.

DISCUSSION

The nuclear maturation of mammalian oocytes is regulated and enhanced by the different components of the media culture. RA had a positive effect on oocyte nuclear maturation evidenced by improving the rate of oocytes that reached the MII stage.

Hidalgo et al. showed that addition of 9-*cis*-retinoic acid into the IVM medium increased the bovine blastocyst development and hatching rate [18]. Duque et al. showed cytoplasmic competence of bovine oocytes improved in the presence of 9-*cis*-retinoic acid during pre-maturation [19]. Livingston et al., demonstrated that supplementation of 5 μM retinol to the maturation medium may improve the embryonic development of bovine oocytes indicated by their increased blastocyst rate. A significant improvement in the blastocyst development with the 5 μM retinol treatment under atmospheric conditions suggests a beneficial antioxidant effect during embryo culture [20].

RA may act to stimulate cumulus expansion by involving the secretion of a protein called midkine (MK). MK is the product of an RA responsive gene [22]. A series of experiments by Ikeda et al. validated that MK enhances the developmental competence of bovine oocytes via cumulus and granulosa cells. It was also demonstrated that MK suppresses the apoptosis that spontaneously occurs in cumulus cells during the period of IVM of bovine cumulus-enclosed oocytes (CEOs) [6,13,23].

The retinol concentration in human serum is 1–2 mM and fetal bovine serum contains less than 20 nM. Hidalgo et al. obtained improved day 60 pregnancy rates after transfer to recipients of blastocysts derived from oocytes matured with 9-*cis*-RA [18]. This retinoid compound improved blastocyst development and quality (i.e., increased inner cell mass/trophectoderm cell proportions) during maturation [19]. The results are consistent with the findings in bovine oocytes [19] and embryos [24]. Yong et al. demonstrated that all-*trans* retinoic acid has positive effects on goat oocyte nuclear maturation and reduces apoptotic cumulus cells during IVM [25]. Liang et al. showed that 5 nm 9-*cis*-RA was beneficial to the nuclear and cytoplasmic maturation of canine oocytes; they inferred an important role for 9-*cis*-RA during IVM [26].

The supply of retinoids to embryo should be ensured within a physiological range, as the exit from the normal range could exert teratogenic defects due to pleiotrophic activity [19]. It was suggested that the optimal concentration of RA for oocytes was chosen as 5 nm for optimal development of bovine. The present results are also in agreement with previous reports indicating that addition of 5 nm isotretinoin to maturation medium like other RA improves maturation rate and fertilization rate of oocytes.

CONCLUSION

In conclusion, we demonstrated that enriching the IVM medium with isotretinoin improved the IVEP efficiency in cattle. It is likely that the enhanced isotretinoin concentration promotes early embryo development by facilitating the completion of oocyte cytoplasmic maturation, ensuring a normal pronuclear development, and hence improving the cleavage rate and overall embryo yield. The improved embryo quality, observed in the presence of isotretinoin, may be the result of reduced oxidative stress during culture. These results can be of

great help in the future for culture media most suitable for oocyte nuclear and cytoplasmic development.

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CONFLICT OF INTEREST

Authors declare there is no conflict.

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