EFFECT OF RETINOIC ACID ON THE DEVELOPMENT OF WATER BUFFALO EMBRYOS *IN VITRO*

Lilibeth A. Cajuday[1] , Annabelle A. Herrera[2] and Danilda H. Duran[3]

ABSTRACT

Retinoic acid (RA), a vitamin A metabolite, regulates cellular growth, embryonic morphogenesis and differentiation. In the present study, the effect of various doses of RA (0, 1, 3 and 5 µM) on *in vitro* maturation (IVM) of water buffalo oocyte was determined. Nuclear maturation and cumulus expansion of the oocytes were enhanced by RA. Cumulus expansion rates in the treatment groups at 1 (80.67%), 3 (80%) and 5 (86%) µM were significantly different compared with the control (72.02%) and the vehicle (67.50%). The percentage of oocytes reaching the metaphase II stage was significantly increased (P<0.05) in the RA-treated group compared with the control (67.97%) and vehicle (66.46%), with 5 µM RA dose (84.47%) exhibiting the highest rate. RA supplementation improved cleavage rate. Treatment with 5 µM RA significantly improved blastocyst formation rate (38.88%) compared with the control and the vehicle. However, there was no significant difference between the control and RA-treated groups in the number of cells of the resultant blastocysts. The results demonstrated that RA enhanced IVM of oocytes to positively influence the development of water buffalo embryos after *in vitro* fertilization.

Keywords: buffalo, cumulus expansion, embryo, retinoic acid, *in vitro* maturation

INTRODUCTION

In vitro embryo production (IVEP) technology in water buffalo has reached considerable success in the last decade and has greatly improved over the years leading to high blastocyst yields (Gasparrini *et al*., 2006) and production of offsprings (Neglia *et al.,* 2004; Hufana-Duran *et al.,* 2004 and 2005; Huang *et al*., 2005). However, IVEP technology in buffalo is still sub-optimal compared with that in cattle (Hufana-Duran, 2008). Meanwhile, the IVEP technology in cattle has become a commercial enterprise, providing avenues for the use of salvage materials from genetically infertile females as well as large-scale embryo production from

[1]Biology Department, College of Science, Bicol University, Legazpi City 4500, Albay, Philippines (email: lacajuday125@yahoo.com); [2]Institute of Biology, College of Science, University of the Philippines, Quezon City, Philippines; [3]Reproductive Biotechnology Laboratory, Philippine Carabao Center, Muñoz, Nueva Ecija, Philippines.

slaughterhouse-derived materials (Hasler, 1998). Generally, in livestock industry, production of embryos using *in vitro* systems represents a low-cost alternative to production of embryos *in vivo*.

One major intrinsic and limiting factor in water buffalo IVEP research is the very low number of available oocytes recovered through ovum pick-up (OPU) or from slaughterhouse-obtained ovaries. Previous studies confirming low efficiency in the development of embryo (Totey *et al.,*1992 and 1993; Cruz *et al*., 1991; Duran *et al.,*1997), low fertilization and development rates (Madan *et al.,* 1994; Palta and Chaunan, 1998) from oocytes matured and cultured *in vitro* made this even worse. These facts confirmed that *in vitro* maturation (IVM), which is the first step in IVEP, is very critical and its success is influenced to a large extent by IVM conditions (Brackett and Zuelke, 1993). Therefore, optimizing the system for water buffalo oocyte IVM will remain a great challenge.

It is during the period of IVM where the oocyte acquires its developmental competence, which refers to the potential of the oocyte to undergo maturation, fertilization, development into the blastocyst stage and, as a final outcome, to produce live offspring (Trounson *et al.,* 2001; Sirard *et al.,* 2006). This is achieved gradually and progressively *in vivo* following a coordinated series of structural and functional changes in the gamete and surrounding cells. In mammals, it is during the course of antral folliculogenesis where the oocyte gradually and sequentially acquires meiotic and developmental competence (Eppig, 1994; Gilchrist *et al.,* 1997).

Defining the culture conditions necessary to support normal oocyte maturation provides a new approach to understanding the factors that regulate normal growth and development of a viable oocyte. One way to attain this is to investigate the culture medium most suitable for oocyte nuclear and cytoplasmic development. During the last few years, the evaluation of culture medium for bovine oocytes has progressed towards more defined conditions having different media investigated for maturation and culture of oocytes and each yielding variable results (Gliedt *et al*., 1996). Consequently, the regimen and protocol for maturation of oocytes *in vitro* vary and, therefore, continue to undergo modifications and refinements to mimic the *in vivo* condition.

Retinoic acid (RA), a metabolite of vitamin A, is an important regulator of cellular growth, embryonic morphogenesis and differentiation (Morris-Kay and Ward, 1999) by influencing the expression of certain genes (Mangelsdorf *et al*., 1994). RA is also essential for ovarian steroid production and oocyte maturation (Ikeda *et al.,* 2005).

Livingston *et al*. (2004) demonstrated that high doses of RA promote blastulation in bovine embryo under extreme atmospheric oxygen conditions due to its beneficial anti-oxidant effect during embryo culture. On the contrary, several studies using murine models have demonstrated a potential adverse effect of RA when administered in excess *in vitro* during the blastocyst stage, such as developmental retardation, inhibition of cell proliferation, induction of apoptosis (Huang *et al*., 2001, 2003 and 2006) and increased production of reactive oxygen species (Santos-Guzman *et al*., 2003).

The effects of RA on oocyte IVM and subsequent blastocyst development

have been demonstrated in domestic animals such as cattle, but not in water buffalo, under physiological culture conditions. Hence, this study was undertaken to validate existing reports on the effects of RA in improving the developmental competence of water buffalo oocytes *in vitro*. To date, there is scarce information on the effect of RA on the production of water buffalo embryos *in vitro* and its effect on cumulus-oocyte complexes' cytoplasmic and nuclear maturation. Thus, in the present work, the effect of RA during maturation within the cumulus-oocyte complex (COC) and after *in vitro* fertilization were analysed. The study also determined the optimum dose of RA on the *in vitro* production of water buffalo embryos.

MATERIALS AND METHODS

Unless specified, all chemicals and reagents used were embryo tested and obtained from Sigma Chemical Co., St. Louis, MO, USA.

Experimental design

Oocytes were divided into five treatment groups: Treatment 1 = Standard Culture Medium (SCM, control); Treatment 2 = SCM + 0.1% ethanol (vehicle); Treatment 3 = SCM + 1 µM RA; Treatment 4 = SCM + 3 µM RA; and Treatment 5 = SCM + 5 µM RA. The concentration of RA was based on previous findings that developmental competence of bovine oocytes was enhanced by 1-5 µM RA (Vahedi *et al*., 2009; Thiyagaran and Valivittan, 2009). For the nuclear maturation experiment, three trials were undertaken. For each trial, data were pooled from two culture plates. For the blastocyst production experiment, three trials were performed and for each trial, data were pooled from several culture plates.

Ovary collection and oocyte aspiration

The ovaries collected from slaughtered riverine water buffaloes of various ages and reproductive status were placed in a thermoflask containing 0.9% NaCl with 100 U penicillin/ml and 100 µg streptomycin/ml at 15-20°C and transported to the Reproductive Biotechnology Laboratory of the Philippine Carabao Center, Muñoz, Nueva Ecija. In the laboratory, the samples were washed thrice in freshly prepared saline without antibiotic. Thereafter, visible follicles (2-8 mm) were aspirated, except those that were atretic as described by Hufana-Duran *et al*. (2004) with the aid of an 18-gauge needle attached to a 10 ml sterile disposable syringe.

Oocyte selection and *in vitro* maturation (IVM)

The collected follicular fluid aspirates were pooled in 15 or 50 ml (depending on the number of ovaries) sterile centrifuge tubes and allowed to settle for 15 min. The selection and classification of oocytes were performed as described by Duran *et al.* (2008) under a stereo microscope and only those cells categorized as Grade A (with ≥5 layers of cumulus mass) and Grade B (with 2-5 layers of cumulus mass) were chosen for the study.

The COCs were washed with the SCM described by Atabay *et al*. (2006). The SCM was primarily TCM 199 (Earle's salts with 25 mM HEPES, Gibco-BRL,

Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 0.2 mM pyruvate, 50 µg follicle stimulating hormone (FSH) /ml, 50 µg gentamycin/ml, 10 ng epidermal growth factor (EGF, Invitrogen)/ml and 1 µg estradiol/ml. Prior to IVM, all-trans RA (50 mg) was dissolved in 1.667 ml ethanol yielding a 100 mM solution, which was later aliquoted and stored in the dark at -20° C.

COCs were incubated for 22-24 h (using 5-10 COCs per 50-100 µl of maturation medium) in tissue culture dishes (35 mm x 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 39°C.

Evaluation of nuclear maturation

After *in vitro* incubation, the oocytes were assessed for nuclear maturation as previously described (Hufana-Duran, 2008). Briefly, *in vitro*-matured COCs were exposed to 0.2 % hyaluronidase for one min to loosen the cumulus cells and vortexed for 2-3 min to completely free the oocyte from the surrounding cumulus cells. Thereafter, the denuded oocytes in culture dish were washed in modified phosphate buffered saline (mPBS) and fixed in aceto-ethanol (1:3, v/v) solution at 4-5°C overnight. After fixation, the oocytes were mounted on clean glass slides, dehydrated with absolute ethanol, stained with 1% aceto-orcein and destained with acetoglycerol (glycerol:acetic acid:distilled water = 1:1:3 v/v). The prepared slides were examined by light microscopy and the oocytes were classified as germinal vesicle (GV), nucleus enclosed by a membrane; germinal vesicle breakdown (GVBD), with disintegrated nucleus and condensed chromosomes; metaphase I (MI), with spindle formation; or metaphase II (MII), with extrusion of the second polar body stage (Kulbelka *et al*., 1988).

Evaluation of cumulus expansion

After 22-24 h of IVM and before the IVF procedure, the morphological appearance of the oocytes was evaluated based on the degree of expansion of the cumulus cells. Oocytes were graded as 0 – not expanded (only outer layer of cells was loosened) or 1 – expanded (cumulus cells were greatly loosened and swollen).

***In vitro* fertilization (IVF) and *in vitro culture* (IVC) of presumptive zygotes**

In vitro matured oocytes were fertilized with frozen-thawed spermatozoa as described by Hufana-Duran *et al.* (2004). Briefly, frozen semen from a single water buffalo bull was thawed at 37°C and added to a sterile centrifuge tube with 8 ml of pre-incubated Bracket and Oliphant (BO) solution (Bracket and Oliphant, 1975) containing 1.25 mM Na-pyruvate and 13.9 mM glucose. Thereafter, the semen suspension was centrifuged for 8 min at 800 g and the supernatant discarded to leave a pellet which was later diluted with BO solution to adjust sperm concentration. Ten µl of the sperm suspension was added to pre-equilibrated 10 µl fertilization droplets consisting of BO medium with 6 mg/ml bovine serum albumin (BSA) and 5 mM theophylline giving a final concentration of 1 x 10^6 sperm/ml, 3 mg/ml BSA and 2.5 mM theophylline. Five to ten *in vitro* matured oocytes were transferred to each fertilization droplet after washing thrice in sperm-free IVF

droplets. *In vitro* fertilization was accomplished by incubating oocytes and sperm cells together in pre-equilibrated fertilization droplets for 6-10 h at 39°C in 5% CO_2.

After the sperm-oocyte co-culture period, the presumptive zygotes were removed from the IVF droplets, denuded of cumulus cells by repeated pipetting, washed several times in pre-warmed culture medium, then transferred into IVC droplets made of modified synthetic oviductal fluid (mSOF) added with 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 for 7-10 days and examined under a stereomicroscope after 26-48 h for cleavage rate and at 7^{th} to 10^{th} day for blastocyst development.

Blastocyst quality assessment

Embryo development was assessed on the 7^{th} to 10^{th} day of IVC. Embryos that developed to early blastocyst, expanded blastocyst, and hatched blastocyst were counted and expressed as percentages. To assess blastocyst quality, only blastocysts that reached the expanded stage were used in examining the number of cells. The number of cells was determined by Giemsa (3%) staining after fixation of the embryos on a glass slide. The cells were examined and counted under an Eclipse E200 light microscope (Nikon UK).

Statistical analysis

Data were expressed as mean±S.E. and analyzed by ANOVA followed by Tukey's honest significance difference (HSD). A probability of $P \leq 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

A total of 564 grades A and B oocytes aspirated from 686 ovaries were matured *in vitro* and evaluated for cumulus expansion, nuclear maturation, cleavage and blastocysts development and quality after IVF. Results are shown in the Table.

Supplementation of RA in the maturation medium improved the percentage of oocytes reaching MII stage, and is consistent with cumulus expansion, cleavage and blastocysts development rates. The quality of the resultant blastocysts was also superior in the RA-supplemented group though not significantly different between the treatment groups. The results are consistent with the findings in bovine oocytes (Duque *et al.,* 2002) and embryos (Lima *et al*., 2005). Several studies in the bovine species (Lima *et al*., 2004; Lawrence *et al*., 2004; Hidalgo *et al*., 2003; Livingston, 2004; Ikeda *et al*., 2006) confirmed the action of RA in enhancing embryonic development *in vitro*. A significant difference was observed between the control and vehicle and oocytes treated with 5 µM RA suggesting that 5 µM is the optimal concentration of RA for oocytes IVM for optimum development in water buffalo.

Water buffalo oocytes exposed in medium without RA had significantly lower rate of cumulus expansion compared with all the RA-treated groups. However, COC expansion among RA-treated groups was not significantly different suggesting that the effect of RA on cumulus expansion is not dose-dependent. RA may act to stimulate cumulus expansion by involving the secretion of a protein called midkine

Table. Effect of retinoic acid (RA) on nuclear development, cumulus expansion, cleavage and blastocyst development and quality in water buffalo oocytes.

Treatment, RA conc. (µM)	Metaphase II development rate % (n)	Cumulus Expansion % (n)	Cleavage, %	Blastocysts Dev., %	Blastocysts Cell Count
Control	70.0+2.8^{a} (32)	72.0±1.0^{a} (72)	60.1±2.0^{a}	20.3±1.2^{a}	91.2±6.4^{a}
Vehicle	66.5±3.7^{a} (30)	67.5±1.7^{a} (74)	62.5±1.4^{a}	21.2±1.5^{a}	90.4±8.1^{a}
1 µM RA	75.9±2.1^{ab} (30)	80.7±4.4^{b} (85)	70.4±3.1^{ab}	31.1±4.8^{a}	107±10.2^{a}
3 µM RA	78.3±1.7^{ab} (34)	80.0±1.9^{b} (90)	70.0±1.9^{ab}	26.7±6.6^{a}	96.8±6.02^{a}
5 µM RA	84.5±2.5^{b} (32)	86.0±1.4^{b} (85)	73.3±3.8^{ab}	38.9±2.9^{b}	96.4±11.3^{a}

Means±standard error in the same column with different superscripts are different (P≤0.05). The oocytes used for analysis of cumulus expansion were also used for the cleavage and blastocysts development rate and quality.
n: number of oocytes

(MK). MK is the product of a RA responsive gene (Kadomatsu *et al.,* 1990). A series of experiments by Ikeda *et al.,* (2000, 2005, 2006) validated the ability of MK in suppressing IVM-induced apoptosis that spontaneously occurs in the COCs during IVM.

The nuclear maturation of mammalian oocytes is regulated and enhanced by the different components of the culture media. In the present study, RA had a positive effect on oocyte nuclear maturation evidenced by improving rate of oocytes that reached the MII stage. This suggests the possible activation of RA-inducible MAP kinase phosphatase gene (Old *et al.,*1995), polyadenylation of m-RNA content in oocytes (Gomez *et al*., 2004), regulation of redox signalling pathways (Olson, 1993; Imam *et al.,* 2001; Ikeda *et al*., 2005) and gene expression of midkine (Gomez *et al*., 2003).

In the present data, the embryo cleavage rate stimulated by 5 µM RA was significantly different from the control (60.10%) and vehicle (62.5%) groups. Cleavage rates were not significantly different among all the RA-treated groups. The role of exogenous RA as a potent growth activator in germ (Morita and Tilly, 1999) and primordial germ cells (Koshimizu *et al.,* 1995) *in vitro* may be reflected in its ability to affect cleavage rate in the water buffalo embryos in the present study. Likewise, retinoids act on cells at the transcriptional level (Ross, 2000); hence, it can modify transcription activity in the COC to influence cytoplasmic maturation and the subsequent capacity of the oocyte to progress in development, including cleavage.

The development-stimulating effects of RA on water buffalo embryos are not only in terms of increase in the rate of produced blastocysts but also on the quality of the embryos as revealed by blastocyst cell count. Although not significant, blastocyst cell count was higher in the treatment group with 1 (107), 3 (96.8) and 5

(96) µM all RA compared with the control and vehicle groups. The present data are consistent with the findings of Hidalgo *et al.* (2003) on the advanced differentiation and total cell number of inner cell mass (ICM) in bovine oocytes matured with RA. Likewise, they are comparable with the total cell number obtained by Thiyagarajan and Valivittan (2009) on water buffalo embryos that were exposed to RA and cultured under controlled atmospheric condition.

One possible mechanism of RA in oocyte maturation as postulated by Ikeda *et al.,* (2000) using cattle as model is the promotion of MK expression. MK is a RA-inducible heparin-binding cytokine (Takada *et al.,* 1997). Since its discovery, MK has been implicated in the regulation of embryogenesis and its expression in various tissues is strictly controlled both spatially and temporally during embryogenesis (Kadomatsu *et al.,* 1990). Furthermore, Ikeda *et al*. (2000 and 2003) demonstrated that the efficacy of a recombinant MK in enhancing the developmental competence of bovine oocytes during IVM was due to its anti-apoptotic activity on the cumulus cells.

The findings of the present study validate the efficiency of RA in improving the developmental competence of oocytes. The non-significant difference in all the RA-treated groups suggests that only low physiological doses of RA are required during IVM.

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