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Production of buffalo embryos using oocytes from *in vitro* grown preantral follicles

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Summary

The present study examines the use of buffalo preantral follicles as a source of oocytes for *in vitro* embryo production. Preantral follicles were isolated from abattoir-derived buffalo ovaries and were grown for 100 days in five different culture systems: (1) minimum essential medium (MEM); (2) coconut water; (3) MEM + ovarian mesenchymal cell (OMC) co-culture; (4) MEM + granulosa cell (GC) co-culture; or (5) MEM + cumulus cell (CC) co-culture. Low growth rates for the preantral follicles were observed when follicles were cultured in MEM or coconut water medium. Moderate growth rates were seen for OMC and GC co-cultures, and high rates of growth were observed when follicles were grown in CC co-culture. The survival of preantral follicles was low in the MEM culture (<25%), but was over 75% in the other culture systems. Oocytes were not recovered from the MEM group, while an oocyte recovery rate of 80–100% was observed when the follicles were cultured with coconut water/somatic cells. Transferable embryos could be produced only with the oocytes obtained from preantral follicles grown in the OMC and CC co-culture systems. This study demonstrates, for the first time, that it is possible to produce buffalo embryos by *in vitro* fertilization of oocytes derived from *in vitro* grown preantral follicles.

Keywords: Buffalo, Embryo, *In vitro* culture, Preantral follicle, Somatic cells

Introduction

Preantral follicles could be an alternative to antral follicles as a source for oocytes used in embryo production. Offspring have previously been produced in mice when using embryos generated with oocytes from preantral follicles (Eppig & Schroeder, 1989), but not in other species. Embryo production from oocytes derived from *in vitro* grown preantral follicles was reported only for pigs (Wu *et al.*, 2001a,b). While for other domestic animals, success has been limited to antrum formation in cultured preantral follicles only, viz. cattle (Gutierrez *et al.*, 2000), sheep (Cecconi *et al.*, 1999) and goats (Huanmin & Yong, 2000). In the buffalo, the ovarian follicular reserve is less in number, coupled with the limitation of a high rate of atresia (Danel, 1987; Aboul-Ela, 2000), both of which are major hindrances in the propagation of superior germ plasm in this species. Hence in recent years, efforts have been made to utilize preantral follicles as a source of oocytes for the production of embryos. To achieve this, previous studies by our group have isolated buffalo preantral follicles (Gupta *et al.*, 2001a) and these were cultured successfully *in vitro* for the first time (Gupta *et al.*, 2002a). In a recent report, (Santos *et al.*, 2006), thousands of preantral follicles were observed in the ovarian cortical sections of fetal buffalo ovaries. Nevertheless, to date, there has been no report of the production of embryos using oocytes retrieved from preantral follicles in this species. Somatic cells, such as cumulus cells or granulosa cells, have been reported to support the growth of oocytes (Nandi *et al.*, 2001) and embryos (Nandi *et al.*, 2002) in buffalos. We hypothesized that somatic cell co-culture would be conducive for the growth of preantral follicles. Here, we describe the work carried out to culture preantral follicles using different culture media (conventional and unconventional) and somatic cells, in order to achieve higher growth and vitality rates and to produce

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embryos utilizing oocytes retrieved from *in vitro* grown preantral follicles.

Materials and methods

All the chemicals were purchased from Sigma-Aldrich, India unless otherwise stated.

Isolation of preantral follicles

Preantral follicles were isolated from abattoir-derived ovaries of mature buffaloes. The combined mechanical and enzymatic method (Gupta *et al.*, 2006) was used to isolate preantral follicles. In short, ovarian cortical slices that had been made with a scalpel blade (no. 21) were incubated in trypsin (1%) at 37 °C for 10 min to retrieve the preantral follicles (150–500 μm) by microdissection with fine sterile needles (26G). During dissection, cortical slices were kept moist with isolation medium comprised of Dulbecco's phosphate-buffered saline plus bovine serum albumin (BSA; 3 mg/ml) and gentamicin (50 μg/ml).

Isolation of mesenchymal cells

The ovarian surface epithelial cells (OSE) were scraped off with a scalpel blade from the surface of a buffalo ovary that was devoid of surface follicles. After the removal of OSE, the ovarian cortex was chopped into small pieces of approximately 1 cm width (Vigne *et al.*, 1994) and washed in isolation medium. The cortical pieces, along with the isolation medium, was repeatedly aspirated with sterile Pasteur pipettes to allow the release of stromal cells and then filtered through sterile 20 μm nylon filters. The filtrate was screened under a stereo zoom microscope for the smallest size category cells, i.e. stromal cells.

Isolation of granulosa cells

Small, thin slices were made from the buffalo ovarian cortex and washed in isolation medium. Next, preantral follicles were dissected out under a stereo zoom microscope and washed three times in preantral follicle isolation medium. Oocytes were removed using a 26G needle and the granulosa cells were aspirated from oocytectomized follicles (Wu *et al.*, 2002).

Isolation of cumulus cells

Oocytes, together with cumulus cells, were aspirated from the surface of medium-sized ovarian follicles from abattoir-derived buffalo ovaries. The cumulus-enclosed oocytes were denuded by repeated pipetting to release the cumulus cells.

Preparation of coconut water solution

Coconut water solution was prepared with coconut water collected under sterile conditions from a single tender coconut. The osmolarity was adjusted to 300 mOsm/l with nanopure water and the pH was adjusted to 6.8 with 5% sodium citrate. The solution was filtered and stored in aliquots at −20 °C until use.

In vitro culture of preantral follicles

Immediately after isolation, the vitality of the preantral follicles was tested by trypan blue staining (Gupta *et al.*, 2002b). Live follicles (265–295 μm in size) without abnormalities and with an intact theca membrane were chosen for *in vitro* culture. The preantral follicles were cultured for 100 days (45 replica samples) in groups of two to three per 50 μl drop of culture medium that had been overlaid with mineral oil and then incubated in an humidified atmosphere at 39 °C, 5% CO_2 in air. The five culture treatments all included the same common additives. The five treatments were: (1) minimum essential medium (MEM; control); (2) coconut water; (3) ovarian mesenchymal cell co-culture (OMC co-culture; $0.05–0.07 \times 10^6$ cells per 50 μl drop); (4) granulosa cell co-culture (GC co-culture; $0.5–0.8 \times 10^6$ per 50 μl drop); and (5) cumulus cell co-culture (CC co-culture; $0.7–1.0 \times 10^6$ per 50 μl drop). The background medium in the three co-culture systems was MEM. The additives used in all the culture systems were steer serum (10%), follicle-stimulating hormone (3 μg/ml), β-mercaptoethanol (10 μM), sodium pyruvate (0.23 mM), glutamine (2 mM), hypoxanthine (2 mM), insulin–transferin–selenium (ITS: insulin 6.25 μg/ml, transferin 6.25 μg/ml, sodium selenite 6.25 ng/ml) and gentamicin (50 μg/ml). Heat-inactivated (at 56 °C for 30 min), filtered (through a 0.22 μm filter) steer serum from the same batch of medium was used for all the treatment groups. The culture medium was replenished every other day with freshly prepared medium. Follicular growth was monitored every 10 days with a micrometer fitted to a microscope.

Retrieval of oocytes from *in vitro* grown preantral follicles

At the end of the culture period, follicular vitality was assessed by the trypan blue staining technique (Gupta *et al.*, 2002b) and recorded as the percentage of follicles surviving out of the total number of follicles cultured, at the end of the culture period. Oocytes were dissected out with fine needles (26G) under a stereo zoom microscope from all the surviving follicles. Oocytes with homogenous ooplasm and at least one layer of compact cumulus cell layer were considered as acceptable for *in vitro* fertilization. The recovery percentage of acceptable quality oocytes was calculated

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as the number of acceptable quality oocytes recovered out of the surviving follicles.

In vitro production of embryos

Production of embryos using the oocytes retrieved from *in vitro* grown preantral follicles was carried out by standard protocol as previously reported (Gupta *et al.*, 2001b) with slight modifications. Groups of six to eight oocytes were cultured in 50 µl droplets of maturation medium, TCM-199 supplemented with steer serum (10%), follicle-stimulating hormone (FSH) (0.05 IU/ml) and gentamicin (50 µg/ml). The droplets were then covered with warm (39 °C) mineral oil and the Petri dishes were placed in a CO_2 incubator (39 °C, 5% CO_2 in air, 90–95% relative humidity) for 24 h. All cultured oocytes were inseminated *in vitro.* Frozen semen from a Murrah buffalo bull (purchased from the Central Frozen Semen Production and Training Institute, Bangalore, India) that was known to have high rates of fertilization and cleavage was used in the present study. Semen from two straws (0.5 ml, 30 million spermatozoa per straw) was washed in Brackett and Oliphant (BO) medium (without BSA) containing 10 µg/ml heparin and centrifuged twice at 500 *g* for 5 min. The sperm were suspended for swim-up in BO medium containing 10 µg/ml heparin and 2.5 mM caffeine. This suspension was placed in 100 µl droplets of BO medium containing 0.5% BSA, 10 µg/ml heparin and 2.5 mM caffeine in a Petri dish, covered with mineral oil, and placed in a CO_2 incubator for 1 h at 39 °C before insemination. The sperm concentration was then adjusted to 8–10×10^6/ml before inseminating the oocytes. The dishes were placed in a 5% CO_2 incubator at 39 °C. After 42–48 h of insemination, presumptive zygotes were evaluated under a stereo zoom microscope for evidence of cleavage. The cleaved embryos, at the 2–4-cell stage or further, were selected for *in vitro* culture study. Resulting embryos were cultured on oviductal cell monolayers of buffalo origin, as standardized previously in our laboratory (Gupta *et al.*, 2001b). Briefly, the cleaved embryos were cultured in a complex co-culture system (TCM-199 supplemented with 10% steer serum (10%) plus 50 µg/ml gentamicin plus 20–40-cell clusters of oviductal epithelial cells) at 39 °C for 8 days.

Statistical analysis

The designs were randomized block arrangements of one control and four treatments each of 45 replicates. In this design, a plot (treatment/replicate) consisted of two to three follicles per culture drop. Statistical analysis was carried out using GraphPad Prism software (GraphPad Inc., USA). The percentage values were subjected to arcsine transformation before statistical analysis. The mean values of different treatment groups were compared by ANOVA followed by the Bonneforni Multiple comparison test. Differences between the mean values were considered significant if the p values were <0.05.

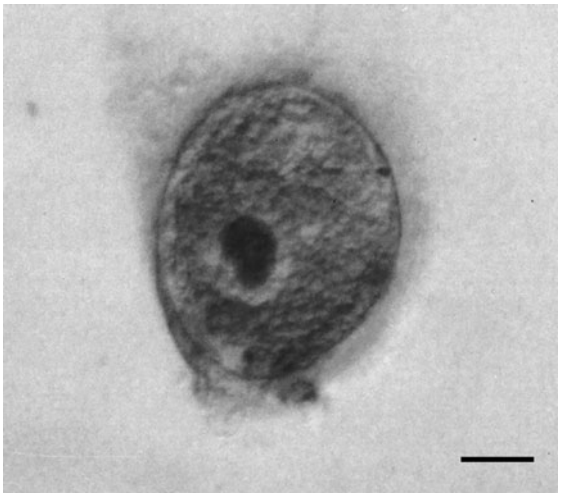

Figure 1 Buffalo preantral follicle (scale bar: 100 nm).

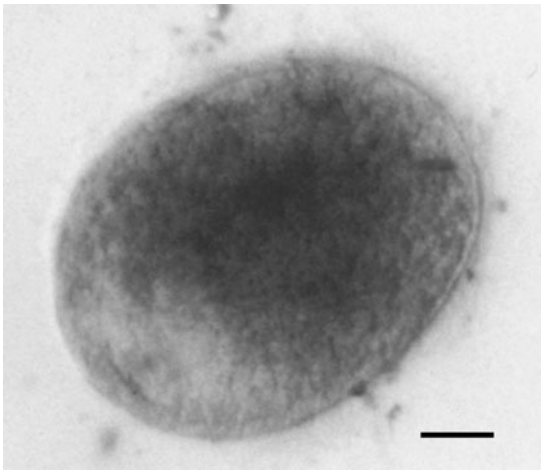

Figure 2 Early antrum formation in buffalo preantral follicle during culture (scale bar: 100 nm).

Results

Among the different treatments there were no significant differences in the size of the preantral follicles at the start (day 0) of culture. After 100 days, the preantral follicles (Fig. 1) showed a significant increase in size in all the *in vitro* culture treatments (Table 1) and antrum formation (Figs. 2 and 3) was observed in the majority of cultured follicles.

The daily growth rate was lowest in the MEM (control) culture group. Culture in the coconut water treatment significantly increased the growth rate to

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Table 1 Effect of *in vitro culture* conditions of preantral follicles on their oocyte development and embryo yields

Culture medium	Preantral follicles			Oocyte recovery rate**% (*n*)	Cleavage rate (%)	Morulae/blastocysts production rate (%)
	Number	Growth rate*	Survival (%)			
MEM + additives (control)	118	1.0 ± 0.06^{a}	21.9 ± 8.16	0.0 ± 0.00^{a} (0)	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
Coconut water + additives	122	1.5 ± 0.05^{b}	85.7 ± 3.51	79.6 ± 0.37^{b} (82)	$28.0 \pm 3.00^{b,d}$	0.0 ± 0.00^{a}
Control + ovarian mesenchymal cells	93	4.0 ± 0.18^{c}	75.6 ± 6.47	81.7 ± 2.71^{b} (57)	45.5 ± 1.15^{c}	12.2 ± 1.10^{b}
Control + granulosa cells	90	4.0 ± 0.18^{c}	51.3 ± 7.48	93.7 ± 6.25^{b} (43)	23.2 ± 0.55^{d}	0.0 ± 0.00^{a}
Control + cumulus cells	101	7.3 ± 0.16^{d}	86.6 ± 12.23	100.0 ± 0.00^{b} (87)	$36.7 \pm 0.05^{b,c}$	11.3 ± 0.85^{b}

All values are in means ± SE.
[a–d]Values in a column with different superscripts differ significantly ($p < 0.05$).
Values are based on 45 replicates/treatment each with two to three preantral follicles per plot.
*Growth rate: growth in µm/day.
**Oocyte recovery rate: percentage of oocytes recovered from survived follicles after culture.

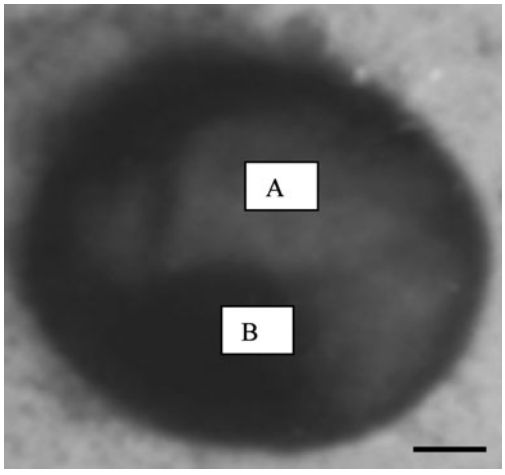

Figure 3 Full antrum formation in buffalo preantral follicle during culture. (A) antrum, (B) oocyte (scale bar: 150 nm).

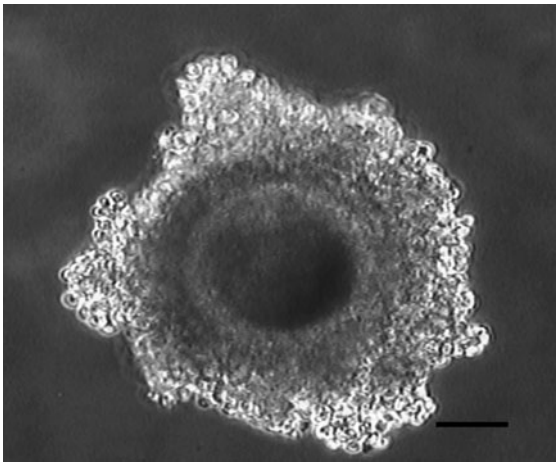

Figure 4 Good quality oocyte collected from *in vitro* grown buffalo preantral follicle (scale bar: 50 nm).

1.53 ± 0.05 µm/day. The co-culture systems resulted in high growth rates of preantral follicles (4–7 µm/day). The highest growth rate was observed when the follicles were co-cultured with cumulus cells. The survival rate of preantral follicles in the MEM culture group was low (<25%), moderate in the GC co-culture (50%) and high (>75%) in the coconut water, OMC and CC co-culture systems. No oocytes were recovered from the control MEM preantral follicle culture system, but oocyte (Fig. 4) recoveries ranged from 79.6 to 100% in the other four treatment groups (Table 1). Cleavage rates for oocytes (Fig. 5) was low for those oocytes recovered from the coconut water and GC-cultured preantral follicles and significantly higher for those from the OMC and CC culture systems. Morulae/blastocysts (Fig. 6) developed from oocytes that had been recovered from the OMC and CC co-culture systems, but not from oocytes from the other treatment groups.

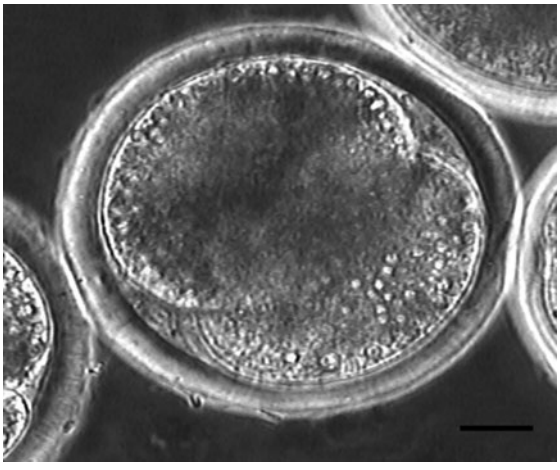

Figure 5 Two-cell embryo produced from oocyte collected from *in vitro* grown buffalo preantral follicle (scale bar: 25 nm).

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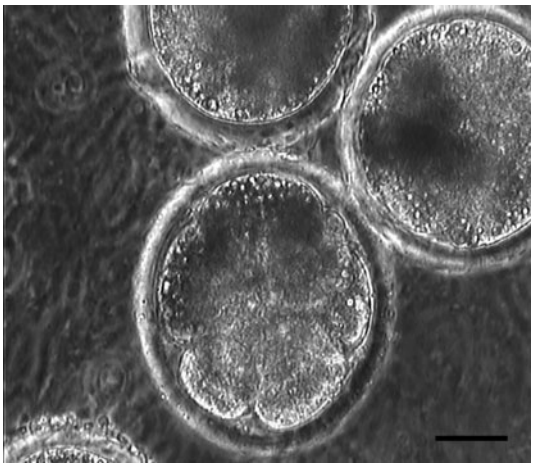

Figure 6 Morula and early blastocyst produced from oocytes collected from *in vitro* grown buffalo preantral follicle (scale bar: 50 nm).

Discussion

The present study demonstrates, for the first time, the production of embryos from *in vitro* grown preantral follicles from buffalos. To date, production of pups with oocytes derived from *in vitro* cultured preantral follicles has been reported only for the mouse (Eppig & Schroeder, 1989), whereas in pigs, embryos could be produced with oocytes derived from preantral follicles (Wu *et al.* 2001a,b). In the present study, ovarian mesenchymal and cumulus cells were found to better somatic cells for preantral follicles co-culture in order to obtain maximum follicular growth, vitality and embryo production.

Use of preantral follicles as a source of oocytes for embryo production may be relevant for buffalo species compared with other domestic animals. This is because of the buffalo's inherent limitations in the reproductive system, such as low germ plasm reserve (Danell, 1987) and a high rate of follicular atresia (Aboul-Ela, 2000), which lead to the low availability of antral follicles that are the normal source of oocytes for embryo production. Several *in vitro* culture systems are used for growth of the preantral follicles in different species. A culture system for buffalo preantral follicles was first reported from our laboratory (Gupta *et al.*, 2002a), in which different growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and vasoactive intestinal peptide (VIP), were used to promote the growth and vitality of preantral follicles. In recent studies in our laboratory (unpublished data), insulin-like growth factor-I (IGF-I) was also tested for its efficacy in promoting the growth and vitality of buffalo preantral follicles. However, in our hands, these factors could not induce sufficient growth for the production of competent oocytes to develop to embryos. In preliminary studies in our laboratory, the co-culture of buffalo preantral follicles with somatic cells, such as ovarian mesenchymal cells, granulosa cells, cumulus cells or oviductal epithelial cells, led to higher growth and vitality rates compared with that of the control (without somatic cell supplementation, unpublished data). Among the four types of cells tested, the three former types of cells produced significantly ($p < 0.05$) better results compared with oviductal epithelial cells. We found that in an earlier study in our laboratory (Gupta, unpublished data) that the growth profile of preantral follicles in relation to time of culture, when using media containing different somatic cells, indicated consistent growth.

Embryo production using oocytes from *in vitro* grown preantral follicles was achieved in mice and pigs. In a study in mice, the growth of preantral follicle led to antrum formation, oocyte growth, ovulation *in vitro* and the birth of offspring (Eppig & Schroeder, 1989; Spears *et al.*, 1994). In 2001, Wu *et al.* (2001a,b) reported the successful production of embryos with oocytes from preantral follicles in pigs with an *in vitro* culture system consisting of FSH, insulin, transferin, L-ascorbic acid and porcine serum.

In bovines, work on this aspect was limited to the antrum formation stage (Gutierrez *et al.*, 2000). The effects of EGF, IGF-I, FSH, and co-culture with bovine granulosa cells on preantral follicular growth were analyzed, and these were found to promote growth and antrum formation by 28 days of *in vitro* culture. Follicular growth was halted by the slower growth of the basement membrane, as growing follicles occasionally burst the basement membrane, extruding their granulosa cells (Gutierrez *et al.*, 2000). This phenomenon was not observed in buffalo preantral follicles in our studies. In another study in bovines (Itoh *et al.*, 2002), in which antrum formation was achieved in cultured preantral follicles, insulin, IGF-I, IGF-II, FSH, and LH all promoted growth and antrum formation in preantral follicles in serum-free medium. Compared with other previous survival studies of preantral follicles in bovine, this is the first report of the survival of preantral follicles after long-term (100 days) preantral follicle culture.

Cellular interactions in the mammalian ovarian follicle between its germ-line and somatic cell components were crucial for its development and function (Eppig, 1991). Somatic cells were reported to secrete certain compounds (Wu *et al.*, 2002) that might help in the growth of preantral follicles. Porcine cumulus and mural granulosa cells produced cumulus expansion enabling factor (Prochazka *et al.*, 1998) and cumulus cells produced a diffusible meiosis-inducing substance (Guoliang *et al.*, 1994). Activin, a granulosa cell product, increased thymidine uptake by mouse preantral follicle granulosa cells *in vitro* (Li *et al.*,

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1995). Granulosa cells also produced c-kit ligand, which regulates thecal cell function, cell proliferation and growth (Parrot *et al.*, 1997). Several growth factors, viz. activin, inhibin, transforming growth factor α (TGFα), TGFβ, EGF, IGF II, FGF, platelet-derived growth factor, and interleukin-6, were found to be secreted or expressed in murine granulosa cells (Vanderstichele *et al.*, 1994**).** Mesenchymal cells express TGFα, TGFβ, keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF), which could regulate granulosa cell growth during ovarian follicle development (Parrot *et al.*, 1994; Nilsson *et al.*, 2003; Kezel *et al.*, 2005). KGF promoted the transition of primordial follicles to primary follicles *in vitro* (Kezel *et al.*, 2005). In the present study, an increased growth for the preantral follicles, when co-cultured with somatic cells, indicated that some factors were secreted into the medium by follicles/somatic cells. As growth of preantral follicles varied in different co-culture systems, the secreting profiles of follicles/somatic cells in culture would also be different. Elucidation of these secreting factors in co-culture merits further investigation.

Addition of β-mercaptoethanol was observed to be beneficial for the growth and vitality of preantral follicles in a study at our laboratory (unpublished data). No report is available regarding the use of this compound in the *in vitro* culture of preantral follicles, although it has been incorporated in the *in vitro* maturation medium (IVM) of buffalo oocytes (Songsasen & Apimeteetumrong, 2002). Embryos developed faster and their quality was better when β-mercaptoethanol was added to the IVM medium. Incorporation of coconut water in the *in vitro* culture medium of preantral follicles was reported in one study of goats (Martins *et al.*, 2004), in which this solution stimulated the development of preantral follicles. However, coconut water has been added *in vitro* to goat ovarian cortical slices (Silva *et al.*, 2004) and promoted the growth of primordial follicles and was also used for the preservation of preantral follicles in goats (Silva *et al.*, 2000) and sheep (Andrade *et al.*, 2002). Coconut water was also found to be suitable for use as a medium for the culture of cattle embryos (Blume *et al.*, 1998). The encouraging results obtained with coconut water solution in our study were probably due to the nutrient composition of this medium, which is rich in proteins, salts, sugars, vitamins, growth factors and plant hormones (Costa *et al.*, 2002). Coconut water contains indole-3 acetic acid (Combarnous & Nunes, 1995), an auxin that binds to certain animal growth factors, such as EGF, in the ovary and enhances their positive effects (Silva *et al.*, 2004). In a recent report (Andrade *et al.*, 2005), indole-3 acetic acid was found to stimulate growth and survival rates in preantral follicles in sheep. Ascorbic acid, a potent anti-oxidant present in coconut water (Leong & Shiui, 2002), might also play an important role in the observed positive effect of this solution in the present study.

In conclusion, the present study demonstrates that embryos could be produced using oocytes derived from *in vitro* grown buffalo preantral follicles. Ovarian mesenchymal cells and cumulus cells can be used effectively for co-culturing with the preantral follicles in order to achieve higher growth and vitality rates and for the production of competent oocytes for embryo production. The biochemical characteristics of the factors secreted by somatic cells in co-culture and the elucidation of factors responsible for the development of preantral follicles in culture merit further investigation. Further studies are required to improve the embryo production rates when using oocytes derived from preantral follicles.

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