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Co-culture of buffalo (*Bubalus bubalis*) preantral follicles with antral follicles: a comparative study of developmental competence of oocytes derived from *in vivo* developed and *in vitro* cultured antral follicles

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Summary

The present study was undertaken to examine whether the presence of antral follicles (AFs) affects the survival, growth and steroidogenesis of preantral follicles (PFs) and compare the maturation and developmental competence of buffalo oocytes derived from *in vivo* developed and *in vitro* cultured AFs. Two experiments were carried out. In experiment I, PFs (200–250 μm) were isolated and cultured with or without AFs (3–5 mm) in TCM-199 medium that contained 10% fetal bovine serum (FBS), 1% insulin transferin selenium (ITS), 20 ng/ml epidermal growth factor (EGF), 0.5 μg/ml follicle-stimulating hormone (FSH) and 100 ng/ml insulin-like growth factor (IGF)-I. In experiment II, *in vitro* developmental competence was compared for the cumulus–oocyte complexes (COCs) recovered from *in vivo* developed and *in vitro* cultured AFs. Survival, growth, development of antrum, accumulation of estradiol and progesterone was ($P < 0.05$) higher when PFs were co-cultured with AFs. Developmental competence of both types of follicular oocytes did not differ significantly in terms of maturation and cleavage rate, but morula and blastocyst production rate were ($P < 0.05$) higher with *in vivo* developed AFs as compared with the *in vitro* cultured antral follicular oocytes. In conclusion, co-culture of PFs with AFs supports long-term survival and growth of buffalo PFs and this co-culture system plays a dual role for *in vitro* production of embryos as well as understanding the relationship between developing PFs and AFs.

Keywords: Antral follicles, Buffalo, Embryo, Oocytes, Preantral follicles

Introduction

Growth and development of different type (primordial, preantral and antral) of follicles within ovary are regulated in a coordinated manner. However, during *in vivo* growth most of these follicles gradually become atretic. In the ovary, the medium-sized leading follicle, mainly antral follicles (AFs) secrete many known, unknown growth factors, hormone and other signaling molecules that are probably involved or associated with the growth and development of preantral follicles (PFs). If these developing PFs are isolated and co-cultured with AFs to make use of the PFs for the production of meiotically competent oocytes it would be of great importance. Such a culture system would supply a combination of the unknown and known growth and survival molecules, secreted by leading AFs that affects the follicular components and the enclosed oocyte of PFs. Methods that allow the co-culture of PFs along with AFs for *in vitro* embryo production before they become atretic would enable better utilization of female reproductive potential. In various domestic animal species, developmental competence of follicles in relation to follicle size, estrous cycle stage and the level of atresia influenced

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by other growing follicles, mainly the early AFs, have been reported (Machatkova *et al.*, 2004; Webb & Campbell, 2007; Barkawi *et al.*, 2009). Furthermore, Webb & Campbell (2007) reported that interactions between extra- and intra-ovarian factors determine the follicle fate as well as the quality of the oocyte.

Studies in various domestic species suggested that *in vitro* development of PFs is regulated by cytokines, growth factors and locally produced hormones (Monniaux *et al.*, 1997; Arunakumari *et al.*, 2006; Rajarajan *et al.*, 2006; Sharma *et al.*, 2009a). Insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF) stimulate proliferation and steroidogenesis of granulosa cells cultured *in vitro* in domestic animals (Hemamalini *et al.*, 2003; Mao *et al.*, 2004; Matos *et al.*, 2007; Spicer & Aad, 2007). Studies have established a relationship between follicle size and oocyte competence. Yang & Rajamahendran (1998) reported that oocytes from 2–3 mm diameter acquired a greater rate of embryonic development, whereas Ireland *et al.* (2008) reported that developmental competence of oocytes greater from 8 mm diameter of follicles. Harada *et al.* (1997) reported that 70% of bovine oocytes isolated from early AFs (0.5–0.7 mm in diameter) showed normal morphology after 8 days of culture. However, using a similar follicle culture system, Miyano *et al.* (1998) reported that only 5% of the oocytes showed developmental competence to the blastocyst stage after IVF. These studies suggested that oocytes harvested from early AFs can grow, and acquire meiotic competence *in vitro.* Fouladi Nashta *et al.* (1998) reported that culture of intact AFs for 24 h is an alternative method for the maintenance of bovine oocytes in meiotic arrest and these oocytes acquire better developmental competence *in vitro.* Previously we showed that collagen gel was a novel and efficacious 3D microenvironment for the extended culture of buffalo PFs (Sharma *et al.*, 2009a). To the best of our knowledge, there is no report on co-culture system for PFs with AFs in domestic animals. Hence, the present study was designed to examine whether the presence of AFs affects the survival, growth and steroidogenesis of PFs and compare the maturation and embryonic developmental competence of buffalo oocytes derived from *in vitro* cultured and *in vivo* developed AFs.

Materials and methods

Chemicals

All the chemicals used in this study were purchased from Sigma Chemical (St Louis, MO, USA), unless otherwise indicated.

Ovaries

Buffalo ovaries from random stages of the estrous cycle were collected from a local abattoir immediately after slaughter and transported to the laboratory within 2 h. During transit, they were maintained at 25–30°C in 0.9% normal saline fortified with antibiotics. Upon arrival at the laboratory, ovaries were freed from ligaments and rinsed several times in pre-warmed phosphate-buffered saline (PBS) supplemented with antibiotics (75 mg/l penicillin-G, 50 mg/l streptomycin sulfate).

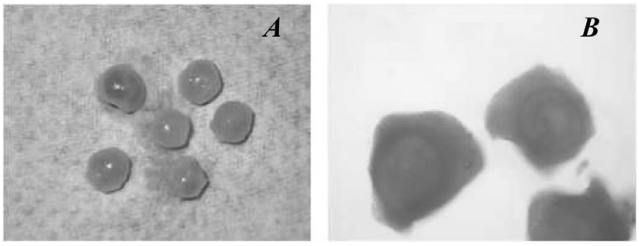

Figure 1 Group of freshly isolated follicles. (*A*) Antral follicles. (*B*) Preantral follicles.

Isolation and selection of PFs and AFs

Under sterile conditions, fine cortical slices (~0.5–1.0 mm) were cut from the ovarian surface using a surgical blade and placed in follicle collection medium supplemented with sodium pyruvate (2 mM), glutamine (2 mM), bovine serum albumin (BSA, 3 mg/ml) and antibiotics (75 mg/l penicillin-G, 50 mg/l streptomycin sulfate) at room temperature. Preantral follicles (200–250 μm diameters) were isolated by micro-dissection method as described previously (Sharma *et al.*, 2009b). Medium-sized AFs (3–5 mm diameter) were isolated by cutting the ovary into two halves and removing the connective tissue around AFs with the help of a pair of fine forceps and surgical blade. Based on the morphological criteria as described by Kruip & Dieleman (1982) for AFs, including brightness, translucency, lack of free particles and the presence of extensive vascularization (observed under stereozoom microscope), cells were selected for the *in vitro* culture. Healthy PFs selected for culture were characterized by at least two granulosa cell layers and a centrally located oocyte. For each of the culture days, 20–25 healthy PFs and 10–12 AFs were isolated from 4–5 ovaries (Fig. 1).

Experimental design

Experiment 1

To determine whether the presence of AFs affects the survival, growth and developmental competence of PFs, mechanically isolated PFs from buffalo ovaries were cultured with or without AFs to understand the functional relationship between AFs and PFs.

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Experiment 2

In experiment II, the maturation and embryonic developmental competence of buffalo oocytes recovered from *in vitro* cultured (experiment I) and *in vivo* developed ovarian AFs (used as control) was compared. Collected COCs from both types of follicles were subjected to *in vitro* maturation (IVM), *in vitro* fertilization (IVF) followed by *in vitro* culture (IVC) to access the embryonic developmental competence.

In vitro culture of AFs and PFs

The basic culture medium was HEPES-buffered (5 mM) tissue culture medium-199 (TCM-199) supplemented with sodium bicarbonate (26 mM). All selected PFs were cultured with or without AFs in TCM-199 supplemented with 10% fetal bovine serum (FBS), 1% insulin transferin selenium (ITS) liquid medium (1 mg/ml insulin, 0.55 mg/ml transferrin and 0.5 μg/ml sodium selenite), EGF (20 ng/ ml), follicle-stimulating hormone (0.5 μg/ml FSH) and insulin-like growth factor-I (100 ng/ml IGF-I). Selected PFs with or without AFs (1 AFs : 3 PFs) were placed in 1 ml of culture medium in four-well plastic tissue culture dish (Nunc, Denmark), overlaid with 1 ml of embryo-tested lightweight mineral oil. These culture dishes were placed in a humidified incubation chamber (Thermo Forma, USA) at 38 ± 1°C in a maximum humidified atmosphere having 5% CO_2 in air. Half of the medium was replenished by an equal volume of fresh medium on every third day. The replaced medium was stored at –30°C for steroid measurement.

Morphological evaluation of *in vitro* cultured PFs with or without AFs

In vitro cultured PFs were evaluated mainly for folliculogenesis (survival, growth, development of antrum), oogenesis (follicular oocyte diameter) and steroids production (estradiol and progesterone). The morphology of each follicle was evaluated every other day using a phase contrast inverted microscope (CKX 41; Olympus, Japan). *In vitro* growth of PFs were monitored by measurement of PFs and their oocyte diameter using Image J 1.33U software (National Institutes of Health, Bethesda, Maryland), based on a calibrated ocular micrometer on 0, 3, 6, 10, 20 and 30 days of culture. Eccentric displacement of the oocyte within the follicles was considered to be an indicator of *in vitro* follicular development. After 10 days of co-culture, AFs were removed from culture medium and the follicular wall was punctured to collect the oocytes for *in vitro* embryo production. The culture period of PFs was extended up to 30 days to examine the influence of AFs on the growth, survival and *in vitro* development of PFs in relation to long-term culture. PFs were stained with 0.5% (w/v) trypan blue for 5–10 min at the end of culture period to evaluate their viability. Follicles that exhibited breaks in the basement membrane, or shrunken or separated follicular cells, were considered as degenerated and were discarded.

Measurement of steroids

Estradiol and progesterone was estimated in the spent culture medium of PFs, AFs + PFs and AFs, which were collected on days 3, 6 and 9 of culture using commercially available radio-immunoassay kits (Immunotech, Czech Republic). Assays for steroids were carried out as per the manufacturer's protocol using duplicate samples. The analytical sensitivities of the progesterone and estradiol kits were 0.02 pg/ml and 4.5 pg/ml, respectively. Inter- and intra-assay variation coefficients for progesterone were 8.5% and 4.5% and for estradiol 11.2% and 12.1% respectively.

Assessment of antrum formation

To access the effect of AFs on antrum development in PFs on days 10 and 30 of culture, PFs were removed randomly from culture dishes and fixed in 10% buffered formalin for 6–8 h at room temperature. The fixed PFs were embedded in paraffin, cut to 5 μm thickness and affixed on to poly-L-lysine-coated glass slide. The sections were stained with hematoxylin and eosin and antrum formation was assessed under phase contrast microscopy (Olympus CKX41, Japan). Six independent experiments were conducted and each group (with or without AFs) had five PFs.

Collection and *in vitro* maturation of oocytes

After 10 days of culture, AFs were removed from the culture medium and follicular fluid was collected by puncture of the follicular wall. For control, fresh buffalo ovaries were used. In detail, all the visible ovarian surface follicles with diameters of 3–5 mm were aspirated to collect the follicular fluid. Cumulus–oocyte complexes (COCs) from both types of follicles were isolated from follicular fluid under a low-power magnification zoom stereomicroscope (SMZ-2, Nikon, Japan). Only oocytes enclosed with compact cumulus cells (3–4 layers) and homogenous cytoplasm was used for *in vitro* maturation (IVM). COCs collected from both follicular system were assigned to IVM (10–12 oocytes in 50-μl droplets) in TCM-199 supplemented with FBS (10%), FSH (0.5 μg/ml), LH (5 μg/ml), 17-β-estradiol (1 μg/ml), sodium pyruvate (0.25 mM) and EGF (20 ng/ml) for 24 h under sterile embryo oil at 39°C and a moist atmosphere of 5% CO_2 in air.

Assessment of nuclear stages

To assess the nuclear maturation, oocytes were taken out randomly at the end of IVM and cumulus

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cells were removed by pronase (0.1%) treatment and transferred to poly-L-lysine-coated glass slides. For fixation, the slides were immersed in methanol:acetic acid (3:1) for at least 24 h prior to staining, the fixative solution was removed by washing two to three times with 100% methanol. The oocytes were stained in 1% (w/v) aceto-orcein and examined under a light microscope (×200 magnification). Oocytes with condensed chromatin were classified to be at the germinal vesicle (GV) stage. Oocytes with diffused chromatin and fragmented nuclear membrane were classified as being at the germinal vesicle breakdown (GVBD) stage. Oocytes with either a polar body or two chromatin masses were classified as being at metaphase II (MII) stage. (Sharma *et al.*, 2007).

Sperm preparation and IVF

Frozen–thawed bull spermatozoa were used for *in vitro* fertilization. In detail, straws of buffalo frozen semen thawed in water at 37°C for 1 min and sperm were washed twice in fertilization (FERT-TALP) medium that contained heparin (10 μg/ml), fatty acids-free BSA (6 mg/ml) and sodium pyruvate (1.0 mM) by centrifugation at 850 *g* for 10 min each. After washing, the sperm pellet was resuspended in 0.5 ml of fresh FERT-TALP. Sperm motility and concentration ($1–2 \times 10^6$) were assessed with a haemocytometer. Droplets of motile sperm suspension were prepared and equilibrated with 5% CO_2 in air for at least 20 min before use. *In vitro* matured oocytes from fresh and cultured AFs were washed in TALP medium and placed in the 100 μl droplets of sperm suspension (10–12 oocytes/droplet) for 18 h in 5% CO_2 in air at 38.5°C.

In vitro culture of embryo

After 18 h co-incubation of sperms and ova, eggs were removed from fertilization droplets and washed in embryo development medium (EDM) supplemented with BSA (3 mg/ml), 10% FBS, sodium pyruvate (0.25 mM), IGF-1 (100 ng/ml), β-mercaptoethanol (0.1 mM) with essential and non-essential amino acids. The washed oocytes (10–12 oocytes/50 μl droplets) were cultured in EDM at 38°C and 5% CO_2 in air for their development until blastocyst stage. The stage of embryonic development was evaluated and the medium replaced every 24 h with fresh medium. Different stages of embryos (2–4 cells, 8–16 cells, morula and blastocyst) from both culture groups were evaluated separately.

Statistical analysis

Data collected from 18–20 experiments on survival, follicle and their oocyte diameter on different days of culture were expressed as the mean ± standard error of the mean (SEM). The data of oocyte maturation and developmental competence were expressed as the percentage (%). Statistical significance was determined using SPSS software for Windows, version 7.5 (SPSS GmbH Software, Munich, Germany) by analysis of variance (ANOVA) followed by Duncan's post-hoc multiple comparison test for proportion. A probability of $P < 0.05$ was considered to be statistically significant. Progesterone and estradiol levels were analyzed over the culture period for 3, 6 and 9 days. These data were analyzed by ANOVA and treatments were further compared by Duncan's multiple range test.

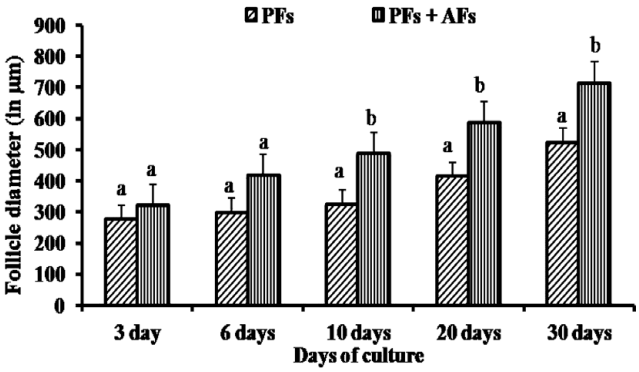

Figure 2 Influence of medium-sized AFs (3–5 mm) on *in vitro* growth of PFs in relation to long-term culture. Values were compared for PFs + AFs vs PFs alone on different days of culture. [a,b]Columns with different superscripts differ significantly at *P*-value < 0.05.

Results

Co-culture of buffalo PFs with AFs improved significantly the *in vitro* growth and survival of PFs. The growth of PFs on day 3 and 6 did not differ significantly, but on day 10, 20 and 30 the growth of PFs increased ($P < 0.05$) in co-culture system (Fig. 2). Following long-term co-culture, up to 22.93 ± 2.84 ($P < 0.05$) PFs had survived as compared with PFs cultured without AFs on day 30 of culture (Fig. 3). The diameter of preantral follicular oocyte increased ($P < 0.05$) when PFs were co-cultured with AFs as compared with PFs alone (Fig. 4).

Accumulation of estradiol in spent culture medium did not differ significantly on day 3 of culture. On days 6 and 9, the concentration of estradiol was ($P < 0.05$) higher in AFs + PFs as compared with AFs or PFs cultured alone (Fig. 5). On day 3, the concentration of progesterone in spent culture medium of PFs cultured alone was lower ($P < 0.05$) in comparision with AFs and AFs + PFs. On days 6 and 9 of culture, the level of progesterone was

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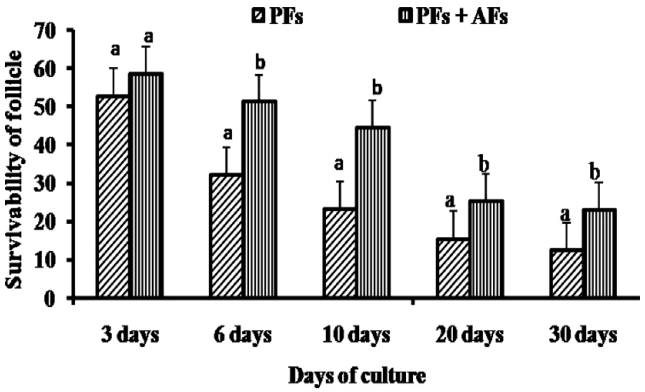

Figure 3 Influence of *in vitro* survivability of PFs cultured with or without AFs (3–5 mm). Columns with different superscripts differ significantly at *P*-value < 0.05. Values were compared for PFs + AFs versus PFs alone on different days of culture. [a,b]Columns with different superscripts differ significantly at *P*-value < 0.05.

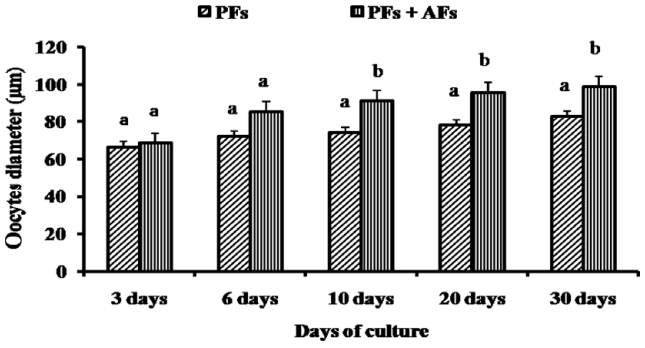

Figure 4 Influence of AFs on oocyte diameter of PFs cultured with or without AFs (3–5 mm) during different culture days. Values were compared for AFs + PFs vs PFs alone. [a,b]Columns with different superscripts differ significantly at *P*-value < 0.05.

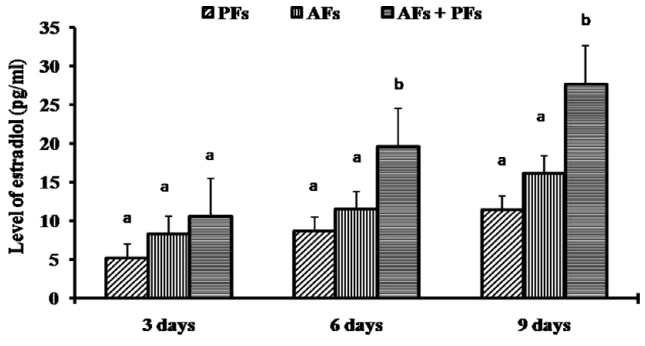

Figure 5 Estradiol production by PFs cultured with or without AFs (3–5 mm) during different culture days. Values were compared for AFs + PFs vs PFs and AFs alone during different culture days. [a,b]Columns with different superscripts differ significantly at *P*-value < 0.05.

higher ($P < 0.05$) in co-culture system of PFs + AFs as compared with PFs or AFs alone (Fig. 6). On day 10 of culture, the antrum formation rate of PFs was lower

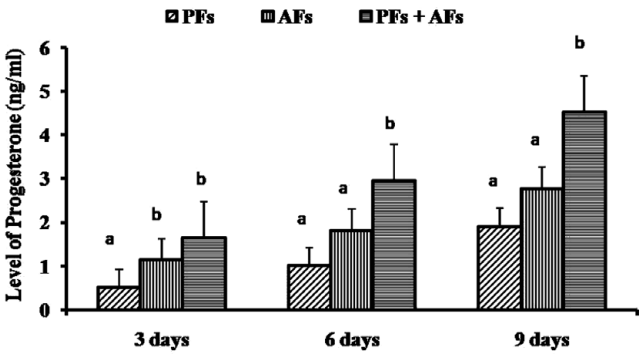

Figure 6 Progesterone production by PFs cultured with or without AFs (3–5 mm) during different culture days. Values were compared for AFs + PFs vs PFs and AFs alone for different culture days. [a,b]Columns with different superscripts differ significantly at *P*-value < 0.05.

($P < 0.05$) as compared with co-culture of PFs + AFs. Under the influence of AFs and in relation to long-term culture, the antrum formation rate was higher but there was significant difference between PFs + AFs and only PFs on day 30 of culture (Fig. 7). Overall follicular viability, growth and antrum formation as observed under a microscope during screening of PFs was positively influenced by AFs.

In total, 185 and 191 good quality oocytes from *in vitro* cultured and *in vivo* developed AFs respectively, were subjected to *in vitro* maturation in gonadotropin-supplemented medium. The maturation rate between these culture groups was not found to be significantly different (Table 1). The developmental potential of embryos obtained after IVM-IVF of oocytes from *in vitro* cultured and *in vivo* developed AFs was compared. No significant difference in cleavage rate (51.78 vs 55.74%) was noted between these two groups of embryos (Table 1). Cleavage rates did not differ significantly up to the 8–16-cell stage embryos in the two different culture groups. However, the development of morula (24.13 versus 42.26%) and blastocyst (12.64% versus 22.68%) was higher ($P < 0.05$) with *in vivo* developed AFs as compared with *in vitro* cultured AFs (Fig. 8).

Discussion

The present study describes the development of a novel and efficacious co-culture system for *in vitro* culture of buffalo PFs along with AFs to establish a long-term culture system and compares oocyte developmental competence derived from two different sources of antral follicle. *In vivo*, the medium-sized AFs (3–5 mm) was recruited from the ovarian preantral

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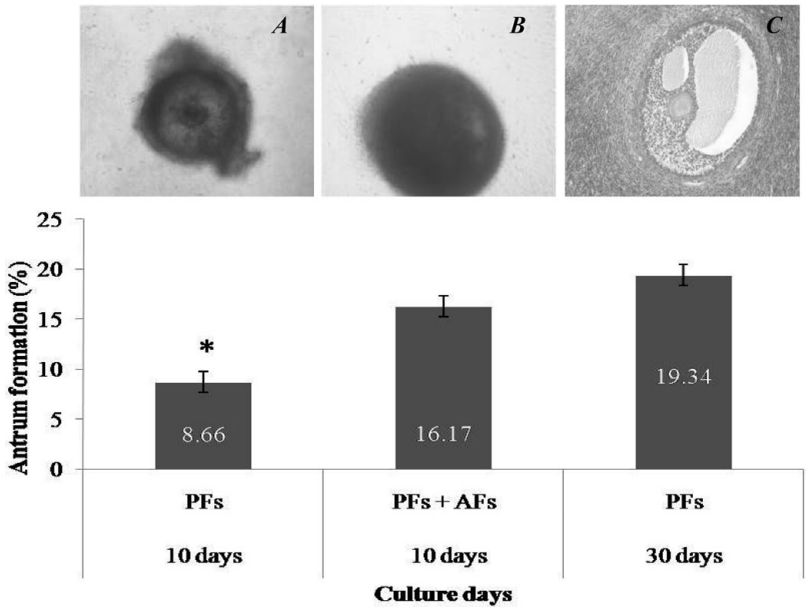

Figure 7 Influence of AFs (3–5 mm) on antrum formation of PFs. Upper panel shows the representative PFs before culture (*A*), antral follicle after 10 days of culture (*B*), and follicle embedded in paraffin and stained with hematoxylin and eosin (*C*), by which the antrum is visible. Bars = 100 μm. In the lower panel, the number in the column is the number of samples that consist of antrum. The percentages were subjected to arcsine transformation and analyzed by ANOVA followed by Duncan's multiple range test. Asterisks on the columns denote significant differences compared with the PFs + AFs and PFs (30 days; $P < 0.05$).

Table 1 Developmental competence of buffalo oocytes collected from *in vitro* cultured and *in vivo* developed antral follicles

Groups	No. of immature oocytes	*In vitro* maturation (%)	Different developmental stages of IVF embryos (%)			
			2–4-cell	4–8-cell	Morula	Blastocyst
In vitro developed AFs	185	168 (90.81%)[a]	87 (51.78%)[a]	61 (70.11%)[a]	21 (24.13%)[a]	11 (12.64%)[a]
In vivo developed AFs	191	174 (91.09%)[a]	97 (55.74%)[a]	67 (69.07%)[a]	41 (42.26%)[b]	22 (22.68%)[b]

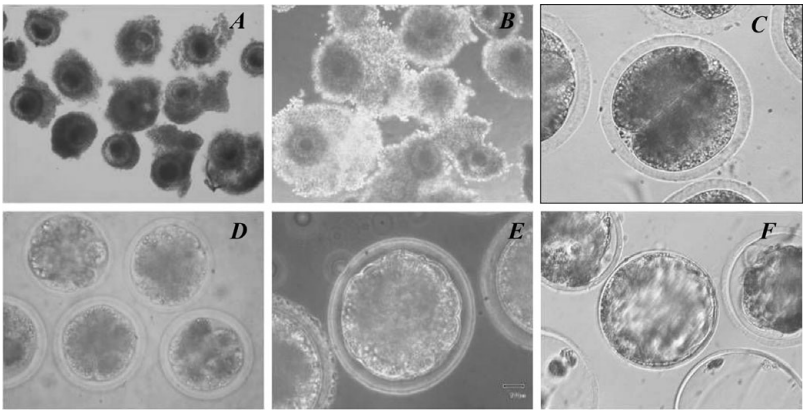

Figure 8 Developmental stages of buffalo embryo produced *in vitro*. (*A*) Freshly collected immature oocytes. (*B*) *In vitro* matured oocytes showing cumulus expansion. (*C*) 2-cell stage of embryo derived from IVF. (*D*) Group of different stages of embryos. (*E*) Compact morula. (*F*) Expanded blastocyst on day 9 of IVF.

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follicle pool, which indicated that, physiologically, AFs might have a role in the development of small PFs. Possibly, the interaction between AFs and PFs is one of the important mechanisms by which a leading follicle is selected. Disconnection of the communication between AFs and PFs by some unknown mechanism(s) might be associated with follicular atresia. Thus, it is possible that some unknown molecules from AFs contribute to survival and growth potential of PFs under *in vivo* conditions. In the present study, PFs of all initial sizes increased in diameter during *in vitro* culture. The growth and survival rate of PFs in the co-culture system did not differ significantly at the beginning of culture (day 1 to day 6), but in the long-term culture (day 10 to day 30) higher growth and survival rate of PFs was observed. Oocyte diameter also increased with increasing duration of culture. In the present experiment, the oocytes reached a diameter of 94.6 ± 1.4 mm after 30 days of culture. Two explanations are possible for the interpretation of the results that showed the impact of AFs on PFs in this study. One possibility is that theca and granulosa cells of AFs produce different factor(s) that affect the growth, survival and development of PFs. Alternatively, theca and granulosa cells of AFs send some factor(s) to granulosa cells of PFs throughout the follicular maturation process, which resulted in enhanced developmental competence of PFs as compared with PFs cultured alone.

The level of progesterone and estradiol production in spent culture medium was stimulated by AFs as observed in bovine (Gutierrez *et al.*, 2000) and ovine species (Campbell *et al.*, 1995**)**. Maximum concentration of estradiol and progesterone was detected in spent co- culture medium of PFs + AFs as compared with PFs alone. In the co-culture system, some signaling molecules secreted by AFs might have stimulated PFs survival, growth and steroidogenesis in a paracrine manner. It has already been established that growth factor interaction with gonadotrophins modulate survival, proliferation and differentiation of follicular cells and oocytes in various animal species *in vitro*, namely mouse (O'Brien *et al.*, 2003), pig (Mao *et al.*, 2004), sheep (Arunakumari *et al.*, 2006), bovine (Wandji *et al.*, 1996; Sudo *et al.*, 2007) and buffalo (Sharma *et al.*, 2009a). Insulin-like growth factor-I stimulates both proliferation and differentiation of granulosa cells and acts as a survival factor (Spicer & Aad, 2007; Sharma *et al.*, 2009a). In the present study, the viability of co-cultured PFs was maintained up to 22% with the help of AFs for at least 30 days. These results suggested that the degeneration of buffalo PFs could be reduced dramatically in the presence of AFs in long-term culture system.

The relationship between follicle size and embryonic developmental competence of their oocytes has been described already, but follicle classes and results were highly variable. Pavlok *et al.* (1992) observed a difference in the developmental competence between oocytes harvested from 2 to 4 mm follicles and 4 to 8 mm. Furthermore, Lonergan *et al.* (1994) reported that blastocyst yield from oocytes that originated from follicles >6 mm was double that of oocytes from 2 to 6 mm. Blondin & Sirard (1995) also reported that oocytes derived from follicles <3 mm had a lower developmental competence, but they could not show differences between 3–5 and >5 mm, and further these results corroborated with Hagemann (1999). In spite of these variable results, it is generally admitted that oocytes from larger follicles have a higher developmental competence than oocytes from smaller ones (Hendriksen *et al.*, 2000). This idea was confirmed in the present study, which showed that oocytes of medium-sized cultured AFs (3–5 mm) had a nearly similar developmental competence as those from *in vivo* developed antral follicles (3–8 mm).

There is some evidence that growth factors secreted by cells stimulate mammalian embryo development (Ghosh & Sengupta, 1998). Many studies have suggested that growth factors in cell-free culture medium improve the blastocyst production rate (Byrne *et al.*, 2002; Sinclair *et al.*, 2003). EGF improves the nuclear maturation and cleavage rate in cattle (Rieger *et al.*, 1998) as well as embryo development in buffalo (Sirisathien *et al.*, 2003; Vikash *et al.*, 2011). Furthermore, growth factors (IGFs, EGF) stimulate mitosis, protein synthesis and reduce apoptosis during embryonic development (Byrne *et al.*, 2002; Sirisathien *et al.*, 2003) through an autocrine or paracrine mechanism (Hardy & Spanos, 2002). The results of our experiments demonstrated that oocytes from both types of follicles matured *in vitro* with gonadotrophin, EGF- and IGF-I-supplemented medium gave an improved maturation rate and better developmental competence with high cleavage, morula and blastocyst production rate as compared with a non-gonadotrophin regimen (data not shown).

The ability to undergo GVBD, progress to, and arrest at the metaphase stage of the second meiotic division is spontaneous when most mammalian oocytes are removed from their AFs and placed in culture. Growing oocytes are not capable of resuming meiosis (undergoing GVBD) and are said to be meiotically incompetent. However, Torner *et al.* (2001) demonstrated that bovine oocytes from 4 to 5 mm follicles required slightly more time to reach anaphase I than those from 2 to 3 mm follicles. In this study, we observed that *in vitro* cultured AFs contain a proportion of poor quality of COCs with a low number of cumulus layers and granulosa cells than *in vivo* developed antral follicular COCs. Probably COCs from *in vitro* cultured AFs performed poorly because

a long culture period had an aging effect that was detrimental to the oocyte developmental competence. So, depending on the selection of the COCs, variable results could be obtained.

In conclusion, the present study provides strong evidence that survival, growth and development of buffalo PFs are regulated by AFs *in vivo* as well as *in vitro*. It is clear that throughout PFs development *in vivo*, AFs support PFs by providing the growth regulators that ensure progression through the protracted growth phase. Embryonic developmental competence of oocytes derived from *in vitro* cultured AFs with a diameter of 3–5 mm has a more or less similar developmental competence to *in vivo* developed AFs. The best option available for the complete growth and maturation of PFs *in vitro* is to develop an extended co-culture system, which will provide a necessary support that closely resembles the ovary *in vivo*. Co-culture of PFs with AFs supports long-term survival and growth of buffalo PFs and this culture system could be used as an alternative source for *in vitro* embryo production.

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