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Effects of ovarian morphology on oocyte quantity and quality, granulosa cells, *in vitro* maturation, and steroid hormone production in buffaloes

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Abstract

The present study was conducted to investigate the effects of ovarian morphology on oocyte quantity and quality, as well as the effect of preincubated granulosa cells (PGCs) on *in vitro* maturation of buffalo oocytes and steroid hormone production. A total of 52 ovarian pairs were grouped into 3 types: Type I (with functional corpus luteum), Type II (with regressed corpus luteum), or Type III (without corpus luteum). The number of follicles and oocytes per ovary were documented. The follicles were classified into 3 groups: <2 mm, 2 to 6 mm, and >6 mm. Oocytes were classified according to their morphology into one of 4 grades (A, B, C, or D) and according to their cumulus compactness into 4 groups (>3 layers of cumulus cells, 1 to 3 layers, partial remnants of cumulus cells, or no cumulus cells). Preincubated granulosa cells were used to investigate their steroidogenic potential with *in vitro* maturation. A greater number of vesicular follicles and aspirated oocytes were found in Type III than in Type II or Type I. The number of Grade A and Grade B oocytes was significantly higher ($P < 0.01$) in number in Type III ovaries compared to other types. Oocytes with >3 layers of cumulus cells had a higher maturation rate than oocytes with partial remnants or no cumulus cells, but had low maturation rate compared to oocytes with 1 to 3 layers of cumulus cells. Besides to the higher maturation rate in compact than denuded oocytes, there was a higher ($P < 0.01$) rate in compact or denuded oocytes when cultured *in vitro* with PGCs than the corresponding oocytes with no PGCs. These maturation rates coincided with a higher ($P < 0.05$) concentration of estradiol-17β when compact oocytes were cultured with or without PGCs compared to denuded oocytes and a higher ($P < 0.05$) concentration of progesterone after culture with PGCs for both compact and denuded oocytes compared to oocytes with no PGCs. In summary, buffalo ovaries with no corpus luteum may result in a higher number of follicles and good oocytes than those with a corpus luteum. Oocytes with an intact cumulus had better maturation than those with partial or denuded cumulus although the denuded oocytes improved their meiotic competence when cultured *in vitro* with PGCs.

Keywords: buffalo, cumulus oocyte complexes, *in vitro*, maturation, ovary.

Introduction

The inherent problem of low oocyte yields from buffalo ovaries (Samad *et al.,* 1998; Chohan and Hunter, 2003) makes warranting the use of *in vitro fertilization (*IVF) procedures questionable. Poor oocyte yield has been attributed to a low number of primordial follicles (10,000 to 19,000) in the buffalo ovary (Samad and Nasseri, 1979; Danell, 1987) compared to cattle (150,000; Erickson, 1966). Despite this major factor, high rates of 70 to 90% for IVM (Nandi *et al.,* 2001; Chohan and Hunter, 2003) have been observed. However, blastocyst development is still very poor and ranges between 10 to 30% (Chauhan *et al.,* 1998; Raghu *et al.,* 2002). In buffaloes, the number and quality of oocytes further decreases during the summer months (Singla *et al*., 1999; Nandi *et al.,* 2001) resulting in fewer oocytes available for IVF studies.

It has been shown that a higher maturation rate could be reached within 24 hours of culture if the oocytes had a compact-cumulus investment (Chian *et al.,* 1995). In addition to the quality of cumulus-oocyte complexes, other factors are also responsible for the success of *in vitro* embryo production. The maturation medium, selection of protein supplements, and hormones for IVM play an important role in the subsequent fertilization and development of mammalian oocytes during *in vitro* culture (IVC; Bavister and Rose-Hellekant, 1992). However, the addition of steroids, especially estradiol and progesterone, improves the completion of maturation (Moor *et al.,* 1980). Consequently, this study aimed to evaluate the effects of ovarian morphology on oocyte quality, as well as the effect of preincubated granulosa cells on IVM and steroid hormones production in Egyptian buffaloes.

Materials and Methods

This study was conducted at the *In Vitro* Fertilization and Embryo Transfer Laboratory, Department of Theriogenology, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Collection of the ovaries

A total of 52 ovarian pairs from Egyptian buffaloes of unknown reproductive history were collected from the local abattoirs of Belbies and Zagazig.

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The ovaries were kept in 0.9% normal saline containing 100 IU/ml Penicillin-G Sodium and 100 mg/ml streptomycin sulfate in a Thermo flask at 37°C and were transported to the laboratory within one hour after slaughter. The ovaries in the laboratory were kept in the same warm saline in sterilized glass Petri dishes (20 cm).

Processing of the ovaries

The collected ovaries were grouped on the basis of the presence or absence of corpus luteum (CL) into 3 types: Type I (with functional corpus luteum), Type II (with regressed corpus luteum), or Type III (without corpus luteum). The number of ovaries and number of follicles per ovary for each ovarian type (I, II, and III) were recorded. Moreover, all the antral follicles were classified for each ovarian type into three different groups according to their diameter (<2 mm, 2 to 6 mm, or >6 mm).

Collecting and qualifying the oocytes

The follicles of different diameters (<2 mm, 2 to 6 mm, or >6 mm) were aspirated with an 18-gauge needle connected to disposable syringe containing 0.5 ml warm culture TCM-199 (Tissue Culture Medium-199) at 38.5º C. The aspirate from each group was then assessed under the stereomicroscope and the oocytes were picked up by narrow pasteur glass pipette and then classified into 4 grades according to their morphology (Gordon, 1995):

Grade-A: Good+ (Score 4, G+): Compact multi-layered cumulus with greater than three layers and a homogeneous ooplasm.

Grade-B: Good (Score 3, G): Compact cumulus of one to three layers with homogeneous ooplasm having a coarse appearance and a darker zona pellucida.

Grade-C: Good- (Score 2, G-): Less compact cumulus with irregular ooplasm containing dark clusters.

Grade-D: Poor (Score 1, P): Nude oocyte or expanded cumulus, irregular ooplasm with jelly like matrix.

The number of oocytes with different grades (A, B, C, and D) for each ovarian type (I, II, and III), and in each follicle diameter (<2 mm, 2-6 mm, and >6 mm) was recorded.

Culture of oocytes in vitro

A total of 238 oocytes were divided into four groups based on their cumulus compactness: including <3 layers (n = 90) or 1-3 layers (n = 56) of cumulus cells, oocytes with partial remnants of cumulus cells (n = 60), or oocytes with no cumulus cells (n = 32). Oocytes were washed twice in warm TCM-199 at 38.5ºC and transferred to 3.5 mm, sterile Petri dishes (15 to 20 oocytes per dish) each containing 2 ml of warm maturation medium (TCM-199 with Earl's salts and L-Glutamine; Gibco Life Technologies, NY, USA) under sterile mineral oil and then incubated at 38.5ºC and 5% CO_2 in air with 95% humidity for 25 to 26 hours (Chohan and Hunter, 2003). The maturation medium was supplemented with 0.5 µg/ml FSH (Folltropin, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) and 10 IU/ml eCG (Novormon, Bioniche Animal Health Canada Inc.), 10% steer serum, 75 ug/ml streptomycin sulfate, 100 IU/ml Penicillin-G Sodium, 10 mM Hepes, and then sterilized by biological millipore filters with 0.22 µm pores.

Collection of granulosa cells

The collection of granulosa cells was carried out by centrifugation of follicular fluid at 2000 rpm/10 minutes. The resulting pellet of cells was washed twice with prewarmed TCM-199 and re-suspended in 400 µl of the same medium and passed several times through an 18-gauge needle to re-disperse the cells (Gordon, 1995). The cells were added to maturation medium (at concentration of 4 x 10^6 GCs/ml) before culture of the oocytes at 72 hours.

Steroidogenic potential of granulosa cells on oocyte maturation

To study the steroidogenic potential of granulosa cumulus cells on buffalo oocyte maturation *in vitro*, a total of 80 oocytes, including compact oocytes (n = 20) with >3 layers of cumulus cells and denuded oocytes (n = 20) were cultured in maturation media with 4 x 10^6 PGCs (counted by a haemocytometer) as described by Gordon (1995) and Nandi *et al*. (2008) and were compared to the same oocytes cultured without PGCs (20 oocytes for compact and 20 oocytes for denuded). Estradiol-17β and progesterone were assayed in maturation media according to Xing *et al*. (1983) by radioimmunoassay using a commercially-available kit (Diagnostic Product Corporation, Los Angeles, CA, USA). Assays of Estradiol-17β and progesterone had sensitivities of 8.0 pg/ml and 0.15 ng/ml, with intra-assays coefficient of variations of 5.3 and 3.9%, respectively.

Evaluation of the maturation stage

After 25 to 26 hours of culture, all oocytes were agitated in 2.9% sterile sodium citrate and cleaned in Dulbecco's saline phosphate buffer to remove remaining granulosa cells. All the oocytes were placed on glass slides and fixed in acetic acid and ethyle alcohol (1:3 v/v) overnight. The oocytes were stained with 2% aceto-orcein (2 gm orcein powder dissolved in 100 ml of 30% glacial acetic acid) and then de-stained with 30% acetic acid (Gordon, 1995). The oocytes were examined microscopically to assess the stage of nuclear maturation including: germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase-I, metaphase-II, and degeneration stages.

Statistical analyses

Analysis of data was performed using SAS analysis system package (Littel *et al.,* 1991). Significant differences between the means were evaluated utilizing Duncan's Multiple Range Test (Duncan, 1955). Treated means were compared at 5% level of probability.

Results

From a total of 52 ovaries collected, 14 were categorized as Type I, 12 as Type II, and 26 as Type III (Table 1). A total of 301 follicles were obtained from the three types of ovaries. Among them, 61, 63, and 177 follicles were from Type I, Type II, and Type III ovaries, respectively. The highest number of follicles and oocytes was observed in Type III followed by Type II and Type I. Depending upon the microscopic morphology of collected oocytes, the highest ($P < 0.01$) number of Grade A and Grade B oocytes was observed in Type III followed by Type II and Type I ovaries.

Table 1. Effect of ovarian morphology on the number of follicles and on the number and quality of oocytes in buffaloes.

Parameter	Type of ovaries (n = 52)		
	Type I	Type II	Type III
No. of ovaries	14	12	26
No. of follicles	61	63	177
No. of follicles/ovary	4.4	5.2	6.8
	Quality of oocytes (n = 270)		
No. of ovaries	14	12	26
Total number of oocytes	51	55	164
No. of oocytes/ovary	3.6	4.6	6.3
Grade A/ovary*	22 (1.57)^{c}	32 (2.67)^{b}	85 (3.27)^{a}
Grade B/ovary	8 (0.57)^{c}	15 (1.25)^{b}	38 (1.46)^{a}
Grade C/ovary	4 (0.29)^{a,b}	5 (0.42)^{a}	4 (0.15)^{b}
Grade D/ovary	17 (1.21)^{a}	3 (0.25)^{b}	37 (1.42)^{a}

*Numbers in parenthesis mean oocytes/number of ovaries.
^{a,b,c}Means within columns are significantly different ($P < 0.01$).

Follicles were classified into three types based on their diameter: <2 mm, 2 to 6 mm, or >6 mm (Table 2). A significantly greater ($P < 0.01$) number of 2- to 6-mm follicles was observed in Type III. Also, a significant difference was observed in the number of follicles <2 mm in Type III ovaries compared to other types, but in the follicles with >6 mm diameter, no difference was observed between the three types of ovaries. When the quality of oocytes was related to follicular diameter, the highest ($P < 0.01$) number of Grade A and B oocytes was observed with 2 to 6 mm follicles compared to other diameters.

Table 2. Mean number of follicles in different ovarian types/ovary and oocytes with different grades/ovary related to follicle size in buffaloes.

Parameter		Follicular groups		
		<2 mm	2 to 6 mm	>6 mm
		Follicles/ovary (n; %)		
Type I	(n = 14)	8 (0.57)^{b}	45 (3.21)^{b}	8 (0.57)^{a}
Type II	(n = 12)	3 (0.25)^{c}	57 (4.75)^{b}	3 (0.25)^{c}
Type III	(n = 26)	33 (1.27)^{a}	133 (5.12)^{a}	11 (0.42)^{b}
		Oocytes/ovary (n = 52) (n; %)		
Grade A		30 (0.58)^{a}	107 (2.06)^{a}	2 (0.04)^{b}
Grade B		5 (0.08)^{c}	53 (1.02)^{b}	3 (0.06)^{b}
Grade C		2 (0.04)^{c}	9 (0.17)^{d}	2 (0.04)^{b}
Grade D		6 (0.12)^{b}	43 (0.83)^{c}	8 (0.15)^{a}

$^{a-d}$Means within columns are significantly different ($P < 0.01$).

A total of 270 oocytes were aspirated from 301 follicles (52 ovaries) in eight replicates. The oocytes characterized by homogenous cytoplasm and varying layers of cumulus cells were used for IVM (238) while the remaining was used to collect cumulus cells. The number of oocytes with >3 layers of cumulus cells appeared to be the highest followed by oocytes with partial remnants of cumulus and then the others. The

IVM rate (Metaphase II) for oocytes with >3 layers of cumulus cells was significantly ($P < 0.01$) higher than oocytes with partial remnants or with no cumulus cells, with small difference compared with the oocytes having 1 to 3 layers of cumulus cells (Table 3). The degeneration rates were significantly higher ($P < 0.01$) in oocytes with partial remnants or with no cumulus cells than the rest of the groups.

Table 3. Effect of cumulus compactness on the ability of buffalo oocytes to mature *in vitro*.

Total oocytes	Oocytes with cumulus compactness (n; %)			
	>3 layers	1 to 3 layers	Partial cumulus	No cumulus
n = 270	94 (34.8)[a]	62 (23.0)[b]	81 (30.0)[a]	33 (12.2)[c]
	Stage of maturation (n; %)			
No. of oocytes (238)	90	56	60	32
GV	4 (4.4)[b]	6 (10.7)[c]	7 (11.7)[c]	5 (15.6)[a]
GVBD	7 (7.8)[a]	4 (7.1)[a]	8 (13.3)[b]	4 (12.5)[b]
Metaphase-I	8 (8.9)[c]	8 (14.3)[b]	10 (16.7)[b]	7 (21.9)[a]
Metaphase-II	58 (64.4)[a]	27 (48.2)[a,b]	5 (8.3)[b]	5 (15.6)[b]
Degenerated	13 (14.5)[c]	11 (19.7)[c]	30 (50.0)[a]	11 (34.4)[b]

[a,b,c]Means within columns and within a row for oocytes with cumulus compactness are significantly different ($P < 0.01$).
GV = Germinal vesicle; GVBD=germinal vesicle break down.

Regarding the level of steroid hormones in the maturation media containing PGCs and its effect on the rate of IVM (Table 4), the level of estradiol-17β was the highest ($P < 0.05$) with compact oocytes cultured with or without PGCs compared to denuded ones. The highest ($P < 0.05$) level of progesterone was detected with the compact or denuded oocytes cultured with PGCs than the corresponding oocytes cultured without PGCs. These levels of hormones were accompanied with higher ($P < 0.01$) maturation rates with compact oocytes either cultured with or without PGCs than denuded oocytes. However, the preconditioned media gave denuded oocytes the ability to mature *in vitro* at a rate near to the group of compact oocytes.

Table 4. The influence of culture with preincubated granulosa cells on the potential of compact and denuded oocytes to undergo meiotic maturation *in vitro* in buffaloes.

Parameter	Compact oocytes (n = 40) (>3 layers of cumulus cells)		Denuded oocytes (n = 40) (no cumulus cells)	
	With PGCs (n = 20)	Without PGCs (n = 20)	With PGCs (n = 20)	Without PGCs (n = 20)
Estradiol-17β (pg/ml)	280.2 ± 1.3[a]	186.1 ± 1.1[b]	128.1 ± 0.6[c]	27.1 ± 0.5[d]
Progesterone (ng/ml)	395.3 ± 2.1[a]	97.9 ± 1.9[c]	227.2 ± 1.1[b]	22.5 ± 0.6[d]
Metaphase II (%)	17/20[A] (85.0)	13/20[B] (65.0)	11/20[A] (55.0)	2/20[B] (10.0)

[a-d]Means with different superscripts in each row are different ($P < 0.05$).
[A,B]Frequencies within groups of oocytes are significantly different ($P < 0.01$).
PGCs = Preincubated granulosa cells.

Discussion

Follicle growth initiation is one of the most important and least understood aspects of ovarian biology and represents a major challenge for experimental study. Changes in the local microenvironment such as the pH and hormone concentrations probably occur as the follicles evolve into the primary stage, but these are probably effects rather than causes (Samad and Nasseri, 1979; Danell, 1987; Datta and Goswami, 1998; Webb *et al.,* 1999). In cattle, there are three wave patterns of follicular growth although two waves or sometimes four waves can occur during the estrous cycle (Savio *et al.,* 1988). Each wave of follicular development is characterized by simultaneous emergence of medium-sized (>4 mm in diameter) growing follicles from a pool of smaller follicles. One of these groups of follicles rapidly emerges as the dominant follicle (7 to 9 mm in diameter) and continues to develop while the others undergo atresia and regress. It usually takes 5 to 7 days for the dominant follicle to develop to ovulatory size (Fortune, 1994; Ginther *et al.,* 1996). Initiation of follicle growth has variously been attributed to: i) hormonal triggers (gonadotropins), ii) stochastic processes (fluctuation in internal signaling molecules), and iii) external inhibitory control from growing follicles (Danell, 1987; Webb *et al.,* 1999). The balance

between the gonadotropins (FSH and LH) and steroids (estrogen and progesterone) might be the important criteria in this process. The greatest number of follicles that were found in Type III ovaries in the present study might reflect the optimum level of gonadotropins and steroids. In type III ovaries, the negative effect of progesterone on the anterior pituitary was not functional. Similarly, the second greatest number of follicles in Type II and the least number in Type I ovaries further confirmed the above statement as regressed corpora lutea were found in Type II and functional corpora lutea in Type I ovaries.

It was reported that progesterone secreted by luteal cells inhibited estrus and caused a negative feedback to the anterior pituitary thus inhibiting FSH secretion (Roy *et al.,* 1972; Hafez, 1993). As a result, the growing follicles regressed and became atretic. In the present study, the effect of progesterone on follicular growth was investigated, and it can be assumed that the higher number of Grade A and B oocytes in Type III and medium to low number of oocytes in Type II and Type I ovaries might arise from the activity of corpus luteum (Shioya *et al.,* 1988). These results further confirmed the findings in this study. A slightly higher number of Grade C oocytes was found in Type II and fewer number in Type I and Type III, respectively. The fewer number of Grade C oocytes found in Type I ovaries might arise from other factors causing atresia. In regard to the number of Grade D oocytes, the unexpected highest value was found in Type III and second highest in Type I although the differences did not reach a significant level. The lowest number of Grade D oocytes was found in Type II ovaries. This discrepancy might come from a lack of recording of the ovarian status as slaughterhouse ovaries were used in the present study.

In 2002, Raghu *et al.,* confirmed that oocytes collected from the follicles of 2 to 6 mm can be used for in vitro maturation, fertilization and subsequent embryo production. Rath *et al.* (1995) collected 2 to 6 mm follicles to determine the efficiency of *in vitro* maturation and fertilization and found acceptable results. The highest number of 2 to 6 mm and <2 mm follicles obtained in Type III ovaries indicated that the recruitment and selection of follicles were functional in the usual manner due to lack of progesterone activity (Roy *et al.,* 1972). Comparatively, fewer numbers of <2 mm and 2 to 6 mm follicles obtained in Type I and Type II explains the reverse effect of progesterone activity. The higher and medium number of follicles >6 mm in Type I and Type III might indicate the growing follicles of the luteal phase but a further study to confirm it would be helpful.

In this study, the significantly highest number of Grade A oocytes followed by Grade B oocytes were observed in 2 to 6 mm follicles. The most notable observation was that a fewer number of Grade A and Grade B oocytes was obtained from follicles >6 mm. Granulosa cells during the maturation period supported the IVM of oocytes to the Metaphase II stage and development to the blastocyst stage in buffalo and cattle (Hashimoto *et al.,* 1998; Singla *et al.,* 1999; Tanghe *et al.,* 2002). In the current study, Grade A and Grade B oocytes were found in significantly greater numbers in Type III ovaries as well as in follicles 2 to 6 mm in diameter. Therefore, 2 to 6 mm follicles from Type III ovaries can be used as a source of oocytes for *in vitro* studies.

Oocyte maturation depends mainly on the important role played by granulosa cells in keeping the oocyte under meiotic arrest, inducing meiotic resumption and by supporting cytoplasmic maturation. These functions have been attributed to their gap junctions and their specific metabolizing capabilities (Tanghe *et al.,* 2002). Physical contact between the oocyte and cumulus cells has been considered necessary for the transfer of nutrients and factors essential for oocyte development (Albertini *et al.,* 2001). However, dissociated cumulus cells have been reported to produce paracrine factors, which initiate resumption of meiosis in denuded oocytes (Downs, 2001). The results of this study showed that buffalo oocytes with a homogenous cytoplasm surrounded by compact layers of cumulus cells had a significantly higher maturation rate than oocytes with partial remnants or no cumulus cells matured with or without additional granulosa cells. The modest increase in IVM rates of co-cultured oocytes in this study can be justified by the fact that paracrine factors produced by the added granulosa cells might have been only transferred to partially denuded oocytes via available gap junctions, whereas such communication seems absent in similar oocytes matured without granulosa cells. This is also evident from the results that addition of granulosa cells not only improved IVM but also rescued oocytes from degeneration whereas more oocytes without somatic cell support underwent degeneration. The present study yielded low numbers oocytes per ovary compared to previous findings in buffaloes (Samad *et al*., 1998). Fewer follicles were found on buffalo ovaries at slaughter during the summer than winter months (Roy *et al.,* 1972), and buffaloes under heat stress produced fewer good quality oocytes than unstressed buffaloes (Singla *et al.,* 1999). The number of oocytes decreased per ovary when selected for IVM on the basis of cumulus morphology. These findings are in agreement to a cumulative number of 34.8% oocytes recovered with >3 and 23.0% with 1 to 3 layers of cumulus cells in the present study. Datta and Goswami (1998) observed a significant decrease in oocyte yield per ovary in buffalo during hot months. In addition, Nandi *et al.* (2001) found a decline in oocytes per ovary when collected during cool (1 to 10^{o}C) and hot (>30^{o}C) months, respectively. The IVM rates also differed between cool (89%) and hot (72%) seasons, but no difference was observed for fertilization, cleavage, and blastocyst development because only matured oocytes were used for *in vitro* fertilization and culture. Our IVM

rates for oocytes with <3 and 1 to 3 layers of cumulus cells were the highest in this study but lower than IVM rates of 84 to 91% previously reported in buffalo (Chohan and Hunter, 2003). However, culture with PGCs in this study increased the IVM rate to the same level. This difference is due to season as well as the use of good quality oocytes. In a previous study, Chauhan *et al.* (1998) found significantly different IVM rates of 85 and 26% for Grade 1 (>5 layers of cumulus cells and homogenous cytoplasm), Grade 2 (>4 layers of cumulus cells and homogenous cytoplasm), and Grade 3 (without cumulus cells and irregular shrunken cytoplasm) buffalo oocytes. Similar observations were recorded for good, fair, and poor quality oocytes in Egyptian buffaloes (Abdoon *et al.,* 2001). Although buffaloes cycle throughout the year, they show a very significant seasonality in breeding in that only 4% come into estrus from April through July (Ishaq, 1957). Considering the low availability of quality oocytes from buffalo ovaries, attempts have been made to utilize the oocytes recovered in denuded form during aspiration in an *in vitro* fertilization system. In a previous study (Das *et al.,* 1997), addition of granulosa cells in maturation medium restored the nuclear maturation of denuded oocytes (64%) close to the compact cumulus enclosed oocytes (66%) but oocytes recovered in denuded form (46%) never reached the same levels of IVM when cultured with no granulosa cells. In another study (Suzuki *et al.,* 1992), buffalo oocytes with compact and dense cumulus cells had a greater IVM rate (67%) than oocytes with a thin cumulus layer (27%) or with small remnants of cumulus cells and poor naked oocytes (3%). These findings are in agreement to the present results but again lack the information about season of the study. Also, lower IVM and IVF rates have been reported (Fukui and Sakuma, 1980; Shioya *et al.,* 1988; Leibfried-Rutledge *et al.,* 1989; Chian *et al.,* 1995; Zhang *et al.,* 1995) for cumulus-free oocytes compared to cumulus-enclosed oocytes in cattle.

The concentration of estradiol-17β was the highest in the medium containing compact oocytes cultured with or without PGCs compared to denuded ones, but the highest concentration of progesterone was detected in the medium containing the compact or denuded oocytes cultured with PGCs than cultured without PGCs. These levels of hormones were accompanied with a higher maturation rate with compact than denuded oocytes. However, the preconditioned media gave the ability of denuded oocytes to mature *in vitro* at a rate near to compact oocytes. A previous study revealed that oocyte maturation and steroidogenesis also occur in cultured follicular cells or follicles in the absence of gonadotrophic hormones (Readhead *et al.,* 1979). However, oocyte maturation is a fundamental step and is dependent on a coordinated interaction between germinal and somatic cells of the follicle (Mattioli, 1996). Granulosa cells from dominant follicles, at least from adult animals, in comparison to all other follicle sizes exhibited a greater number of gonadotropin receptors (Khatir *et al.,* 1997). It was thought that cattle oocytes are stimulated to mature *in vitro* through the addition of granulosa cells from dominant follicles (O´Doherty *et al.,* 1996; Amer, 2002) although no explanation was found by Alm *et al.* (1990) for different oocyte behavior in response to the use of fresh or preincubated granulosa cells.

The supplementation of the culture medium with granulosa cells improves the *in vitro* maturation of bovine oocytes by increasing hormone concentrations, which act as paracrine factors in the culture medium that are mediated by cumulus cells (Bevers *et al.,* 1997) and consequetly enhances the resumption of meiosis that requires synthesis of new preovulatory specific proteins (glycosylated) transmitted by cumulus cells (Hashimoto *et al.,* 1998). The promoting of oocyte maturation with co-culture of cumulus cells *in vitro* attributed to the gradual appearance of proteins and growth hormones secreted by cumulus cells (Izadyar *et al.,* 1999) indicate a paracrine and/or autocrine action of growth hormone on oocyte maturation. These actions also regulate several key granulosa cell enzymes involved in cumulus expansion and maintenance of an optimal oocyte micro-environment (Elvin *et al.,* 1999). The best result of IVM was obtained using natural concentrations of estradiol produced by co-cultured granulosa cells whereas the worst results of IVM occurred at higher concentrations of estradiol supplementation (Mingoti *et al.,* 1995). Regarding concentration of progesterone, granulosa cells cultured *in vitro* showed signs of luteinization as indicated by the greater release of progesterone compared to estradiol (Mayes, 1999). This pattern is characteristic of granulosa cells *in vivo* following the LH peak. Luteinization of granulosa cells *in vitro* may be attributed to the expression of the mRNA coding for the LH receptors (Peng *et al.,* 1991). These previous reports indicated that the culture of buffalo oocytes with granulosa cells *in vitro* can stimulate the maturation of both denuded and compact oocytes by the synergetic action between them acting in a paracrine and/or endocrine pattern.

In conclusion, (i) buffalo ovaries with no corpus luteum resulted in a greater number of follicles and good-quality oocytes compared to those with a functional or regressed corpus luteum; (ii) oocytes with an intact cumulus had better *in vitro* maturation than those with partial or denuded cumulus; and (iii) nuclear maturation can be restored to a substantial number of buffalo oocytes recovered in denuded form or with partial remnants of cumulus cells by addition of preincubated granulosa cells, which is important when dealing with low numbers of buffalo oocytes. Therefore, 2 to 6 mm follicles from ovaries with no corpus luteum can be used as a source of oocytes for *in vitro* maturation, fertilization, and subsequent culture study.

It is suggested that studies using abattoir ovaries may not be truly representative of the potential of the buffalo ovary because mostly aged and underfed animals are slaughtered. Therefore, further studies should be focused on oocytes recovered from animals in good body condition.

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