Involvement of Granulosa Cells in Realization of Prolactin Effects on the Developmental Competence of Bovine Oocytes Matured *in vitro*

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Abstract: The objective of this study was to evaluate the role of granulosa cells (GC) and bovine prolactin (bPRL) on nuclear and cytoplasmic maturation of bovine oocytes and their developmental competence in vitro. The level of apoptosis in GC, the mitochondrial activity and the concentration of intracellular stored calcium ($[Ca^{2+}]_{is}$) in the matured oocytes, as well as morphology and the chromatin status of produced embryos after IVF were analyzed. Cumulus-oocyte complexes were incubated in TCM 199 containing 10% FCS (control group). Oocytes of experimental groups were culture in control medium added by 10^6 GC or 50 ng/ml bPRL or 50 ng/ml bPRL with 10^6 GC (experimental groups). The highest percentage of normal embryos at morulae and blastocyst stages was obtained from oocytes matured in the presence of bPRL and GC (47.9%). The fluorescence intensity of metabolically active mitochondria measured by intensity per oocyte (Em 570) after MitoTracker CMTM Ros Orange labeling was significantly increased in oocytes matured in the presence of 50 ng/ml bPRL and/ or GC (254,3 ± 20,2 µA vs. 119.9 ± 20.4 µA; $P<0.05$). In parallel, the concentration of $[Ca^{2+}]_{is}$ in oocytes matured in bPRL and/or GC , determined by using the fluorophore chlortetracycline, was significantly lower (0,67 ± 0,06 A.U. vs. 1,0 ± 0,07 A.U., $P <0.05$). In addition, it was shown that bPRL decreased the level of apoptosis and the percentage of cells with pycnotic nuclei in GC. In conclusion, it was found that granulosa cells are involved in the realization of prolactin's action on the developmental competence of bovine oocytes matured *in vitro*. [Journal of American Science 2010; 6(9):796-805]. (ISSN: 1545-1003).

Keywords: Granulosa cells -Oocytes - *In vitro* maturation - Prolactin

1. Introduction:

In the past incredible efforts has been made to understand the mechanism underlying follicle growth, differentiation, and several in vitro systems have been developed to enhance *in vitro* embryo production. These protocols were developed in experimental applications to increase the number and quality of ova available for *in vitro* fertilization (IVF). The complexity of ovarian follicles is becoming evident and morphological and functional unit in which development is finally regulated by expression of autocrine and paracrine factors and by interaction between cellular components of the follicles, somatic and germ cells. Follicle cells are essentials for oocyte growth. Indeed the rate of oocyte growth in vitro can be directly related to the number of granulosa cells (Brower & Schultz, 1982). It was reported that supplementation of maturation media with granulosa cells improved cytoplasmic maturation of oocytes. Granulosa cells when cocultured with oocytes may interact with cumulus-oocyte-complexes, and influence developmental competent oocytes (Brower & Schultz, 1982).

Hormonal supplementation to medium is required to promote cytoplasmic maturation of bovine oocytes such that developmentally competent oocytes could be obtained after in vitro maturation (keefer et al.,1993). Prolactin have been shown to enhance developmental competence of *in vitro* matured rabbit oocytes (Yoshimura et al., 1989) and bovine oocytes (Alm et al.,1998). Prolactin interact with its receptors on plasma membrane but does not activate adenylate cyclase like LH or FSH (Brower and Schultz, 1982)

Calcium-ions are considered a plaustile second messenger of PRL, because it induces mobilization of Ca^{2+} from intracellular stores as well as its influx from extracellular space (Doppler, 1994; Vacher et al 1994). The fluorophore chloretetracyclin (CTC) is know from fluorescent complexes which was previously called "membrane bound calcium", since

fluorescence intensity of the complexes was greatly enhanced in the vicinity of membrane (Caswell, 1979).

Apoptosis has been described as specific set transformations which take place at microscopic level associated with cell death. When cell committed to die, it will stop communicating with its neighboring cell/tissue. This evident dying cell detaches itself from the neighboring cells while the chromatin at the nuclear membrane and nucleus fragment become condensed. Cells shrink age as a result of cytoplasmic condensation possibly resulted from loss of water and cross linking proteins. Finally, the cell separated into a number of membranes bound fragments of varying sizes which are called apoptotic bodies (Jolly et al., 1997). Granulosa cell (GC) apoptosis has been widely used to identify atretic follicles, very little evidence was used as a marker for oocytes quality.

Therefore, the objective of this study was to evaluate the role of granulosa cells through (i) the effect of prolactin on the nuclear and cytoplasmic maturation of bovine oocytes and their developmental competence in vitro. (ii) The level of apoptosis and pycnosis in granulosa cells, (iii) the mitochondrial activity and the concentration of intracellular stored calcium (Ca^{2+}) in the matured oocytes, and (iv) The morphology and the number of nuclei in the in vitro produced embryos after IVF was analyzed.

2. Materials and Methods:

Recovery of oocytes and preparation of granulosa cells:

Ovaries from dairy cows were obtained at a slaughterhouse and transported within 2 h to the laboratory in sterile phosphate buffer saline (PBS) maintained at 35 to 37°C. The cumulus–oocyte complexes (COCs) were recovered by slicing the surfaces of ovaries. The oocytes were collected in HEPES TCM 199 with 10% fetal calf serum (FCS). Only oocytes possesses complete, compact, multilayered cumulus and homogeneous ooplasm were used for culture. Before cultivation, the oocytes were washed twice in TCM 199 with 10% FCS plus antibiotics.

Granulosa cells (GC) were isolated from the medium size follicles (3 to 5 mm in diameter) from which COCs were dissected and centrifuged twice for 5 min at 500 g. The final pellet of granulosa cells was resuspended in maturation medium. Co-culture of oocytes and granulosa cells was carried out in Petri dishes in 2 ml of medium at 38.5°C in an atmosphere of 5% CO_2 for 24 h.

In vitro maturation (IVM):

The selected COCs were matured for 24 h at 38.5°C in different treatment groups based on following culture condition

1. Control medium (CM) consists of TCM 199 with Earle's salts, L-glutamine, 25 mM HEPES supplemented with 10% (v/v) heat-treated FCS, and trace amounts of antibiotics (50 IU penicillin, 50 µg streptomycin / ml).
2. CM + GC contained TCM 199 with Earle's salts, L-glutamine, 25 mM HEPES supplemented with 10% (v/v) heat-treated FCS, trace amounts of antibiotics (50 IU penicillin, 50 µg streptomycin /ml), and $1x10^6$/ml granulosa cells (Dendekar et al. 1991).
3. CM + bPRL contained TCM 199 with Earle's salts, L-glutamine, 25 mM HEPES supplemented with 10% (v/v) heat-treated FCS, trace amounts of antibiotics (50 IU penicillin, 50 µg streptomycin / ml), plus 50 ng/ml prolactin (Heleil et al., 2001).
4. CM + bPRL + GC contained TCM 199 with Earle's salts, L-glutamine, 25 mM HEPES supplemented with 10% (v/v) heat-treated FCS, trace amounts of antibiotics (50 IU penicillin, 50 µg streptomycin per ml), plus $1x10^6$ /ml granulosa cells, and 50 ng/ml prolactin.

In vitro fertilization (IVF):

After IVM, matured oocytes from different culture systems were fertilized *in vitro* using frozen-thawed bovine semen. Frozen semen from bulls, with proven high rates of IVF and cleavage was used. Motile spermatozoa were obtained by swim-up separation based on the method of Lonergan et al., (1994). After maturation, the oocytes were transferred to modified TALP medium and most of cumulus was removed mechanically by gentle pipetting. Five oocytes were placed in a 50 µl droplet of fertilization medium (Fert-TALP: 21) and 5-8 µL of final sperm suspension were added to each fertilization droplet to give final concentration of approximately 1 x 10^6 motile sperm/ml in fertilization droplet. Fertilization was carried out for 24 h at 38.5°C under 5% CO_2 in humidified air.

Assessment of fertilization:

At 20 to 24 hours after fertilization, 10% of the oocytes were randomly mounted, fixed in methanol-acetic acid (3:1), stained with 5% Giemsa solution for 10 min at room temperature. Oocytes were examined under a phase contrast microscope at a magnification of 400x. Oocytes were classified as fertilized when a decondensing sperm head with its accompanying tail or 2 or more pronuclei were present in the ooplasm. Ova that were uncleaved were fixed and stained to determine whether fertilization had occurred (Lonergan et al., 1994).

In vitro culture of embryos:

Twenty four hours after IVF, the presumptive zygotes were denuded and transferred to Menezo B_2 medium (Laboratories C.C.D., Paris, France). After IVF, the cleavage rate (number of egg had cleaved to the two- cell stage or beyond at 48h after IVF) was recorded. the cleaved embryos were transferred to previously prepared bovine oviductal epithelial cell (BOEC) monolayer cell line. After 3 days of culture, 40µl of the culture medium was placed with fresh embryo culture medium. On day 8 (day 1 = day of fertilization), the proportion of blastocysts developing at the end of the 8 day culture period were compared among groups.

Evaluation of oocytes and embryos

Nuclear maturation of the oocytes was evaluated using the cytogenetic method described by Tarkowski (1966). Oocytes matured over 24 h were placed in 0.9% sodium citrate in water for 10 min, stripped free of cumulus cells using a dissecting needle, fixed in methanol – acetic acid (3:1), stained with 5% Giemsa solution for 10 min at room temperature, and examined under phase-contrast microscope optics at 250-630x magnification for determination of nuclear status and criteria for maturation were a metaphase II chromatin and extruded polar body as described by Heleil et. al (2001). . The procedure to estimate the chromatin status of embryos was the same as described for oocytes; however, before analysis, the embryos were also incubated for 20 min in 0.1% hyaluronidase. Embryos with two- or multinuclear more blastomeres, blastomeres including pycnotic chromatin, and blastomeres without chromatin were classified as not intact (Tarkowski, 1966).

Assessment of granulosa cells apoptosis and pycnosis

Terminal deoxynucelotidyl transferase mediated dUTP nick and labeling (TUNEL). An In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) was used to detect cell death (apoptosis) in situ with minor modifications to the manufacturer instructions. Procedures were as follows: (1) the drop of Dulbeccos phosphate buffered saline (D-PBS) with GC was collected into a 1.5 mL tube on ice, and GC were separated from follicular fluid and D-PBS by centrifugation (200 x g, 5 min) at 4°C; (2) the cell pellet was re-suspended in 200 µL PBS and centrifuged for 5 min at 200 x g; (3) the pellet was re-suspended and fixed in 4% paraformaldehyde in PBS for 1 h at room temperature; (4) centrifuged in PBS and treated with 0.1% Triton X-100 at 4°C for 2 min; (5) centrifuged in PBS and incubated with TUNEL reaction mixture (50 µL) in a humidified chamber at 37°C for 30 min; (6)10 µg/mL propidium iodide (PI) was added to the TUNEL reaction mixture and stained for 5 min; (7) after centrifugation and resuspension in PBS, the cells were speared on a slide, covered with coverslip and observed under a fluorescence microscope. Four to six fields of each slide were examined on each slide, and percentage of apoptotic cells were calculated from 200 GC as described by Feng, et al. (2007).

For determination of pycnosis in GC the collect pellet of GC was resuspended with 0.9% sodium citrate, fixed in methanol acetic acid (3:1) and stained with Giemsa stain. Smears of GC cells were assessed by microscopic examination and the contribution of the GC with pycnotic nuclei to the total number of granulosa cells (not less than 1000 cells) was calculated and expressed in percent as described by Lebedeva et al. 1998).

Intracellular stored calcium $[Ca^{2+}]_{is}$ assay

A total 87 oocytes were used for estimation, levels of $[Ca^{2+}]_{is}$ in the oocytes on the basis of previously described measurements of the fluorescence intensity of calcium-chlortetracycline (CTC) complexes on irradiation (Denisenko et al., 2005 Kuzmina et al.,1999). The fluorescence intensity was recorded under a luminescent microscope (LUMAM – I – 1, LOMO, St. Petersburg, Russia) equipped with a special photoelectronic multiplier (FEU-39A), an excitation filter (FS1; 6-mm thick), a green analysis filter, an interference light filter (passing maximum wavelength of 520 nm), and a mercury lamp. The excitation wavelength was 380 to 400 nm, and the emission wavelength was 520 nm (slit=10nm).Measurement was performed within 10 sec. Chlortetracycline solutions were prepared in saline (150 mM NaCl, pH 7.0) immediately prior to the experiments. Oocytes were placed in special quartz wells with saline containing 40 µM CTC and were incubated for 5 min in a water bath (37°C). The diameter of the probe used was constant (1 mm), and the diameter of the areas assayed was 100 µm. Irradiation was focused at the center of the oocytes, and the intensity of cell fluorescence was measured as an electric signal. The values of the background emission were subtracted from the obtained magnitudes. The levels of $[Ca^{2+}]_{is}$ were expressed in arbitrary units (AU) of the intensity of oocyte fluorescence.

Fluorescence labelling of mitochondria and measurement of fluorescence intensity

The *in vitro* matured COCs were incubated for 30 minutes in PBS containing 3% bovine serum albumin (BSA) and 200 nM MitoTracker Orange CMTM Ros (Molecular Probes, Oregon, USA) under culture

conditions (Torner et al., 2004). Cell-permeant MitoTracker Orange-fluorescent tetramethylrosamine (M-7510) probe is readily sequestered by actively respiring organelles only depending upon their oxidative activity. Then, the cumulus cells were removed as described above, and oocytes were washed 3 times in pre-warmed PBS without BSA. Oocytes were fixed for 15 minutes at 37 °C using freshly prepared 2% paraformaldehyde in Hank's balanced salt solution. The M-7510 probe contains a thiol-reactive chloromethyl moiety and can react with accessible thiol groups on peptides and proteins to form an aldehyde-fixable fluorescent conjugate that is well-retained after cell fixation over a period of six weeks. After fixation, Oocytes were washed 3 times in PBS, mounted on slides under coverslips, and stored in the refrigerator prior to evaluation by fluorescence microscopy.

An epifluorescence microscope (Jenalumar, Carl Zeiss, Jena, Germany) was used for all experiments. Emission wavelengths were separated by a 540 nm dichroic mirror followed by further filtering through a 570 nm long pass filter (red emission). The mitochondrial aggregation pattern of the bovine oocytes was characterized by observation (up to 500 X magnification) of labeled mitochondria that were oxidatively active. The aggregation patterns were mainly classified as fine (small pixels of fluorescence intensity throughout the cytoplasm), or aggregated (larger linear aggregations of fluorescence intensity).

The fluorescence intensity (µA) was measured using a Nikon Photometry System P 100 (Nikon, Düsseldorf, Germany). Microscope adjustments and photomultiplier settings were kept constant for all experiments. Oocytes were positioned in the plane of focus, and the area of measurement was adapted to the size of the oocyte. The emission intensity/oocyte data was reduced by compensation for the background fluorescence.

Statistics

The data was evaluated by Chi-square test, and all results with $P < 0.05$ were considered to be significant. Three replicates were performed in all experiments. Differences in the intensity of fluorescence of complex membrane-bound calcium-chlortetracycline were tested by ANOVA. Statistical analysis of the fluorescence intensity of mitochondria was conducted using the SAS System for Windows (release 8.02).

3. Results

Experiment I:

To determine the effect of bPRL on the developmental competence of bovine in the presence of GC on developmental competence of oocytes matured *in vitro*. We compared the proportion of matured oocytes (i.e. metaphase II stage), the fertilization and cleavage rates, and the number of morulae and blastocysts after IVM in four different culture systems as shown in Table 1. There were no differences in the percentages of oocytes reaching the metaphase II stage in all groups of experiment. As compared with control, supplementation with PRL or GC alone led to increase in the development to blastocyst stage, but highest level of blastocysts was observed after maturation with bPRL and GC together.

Table (1): Effect of bPRL in the presence or absence of GC on the developmental competence of bovine oocytes (n=750 oocytes)

Culture system	Matured oocytes (M.II) % (n)	Fertilized oocytes % (n)	Cleavage rate % (n)	Morulae/ blastocyst % (n)
TCM 199 ± 10% FCS	78.7 (70/89)	29.6 (29/98)[a]	18.4 (18/98)[a]	0.0(0/98)[a]
TCM 199 ± 10% FCS ± GC	85.6 (77/91)	58.4 (52/89)[b]	39.3 (35/89)[b]	18.0 (16/89)[b]
TCM 199 ± 10% FCS ± 50 ng/ml bPRL	83.3 (65/78)	67.3 (68/101)[c]	41.6 (42/101)[c]	22.8 (23/101)[c]
TCM 199 ± 10% FCS ± GC ± 50 ng/ml bPRL	81.9 (68/83)	76.0 (92/121)[d]	62.0 (75/121)[d]	47.9 (58/121)[d]

Values followed by the different letters are significantly different at P<0.05 (within columns).

Experiment II:

It is conducted to determine the influence of bPRL on apoptosis and pycnosis on the GC as shown in Table (2 and 3). Where GC was examined under a fluorescence microscope after TUNEL and PI staining, all nuclei stained red with PI, whereas only nuclei of apoptotic cells stained green-yellow by TUNEL (Fig. 1, 2). After *in vitro* maturation, the proportion of apoptosis in GC significantly (P<0.05) increased as compared to that at beginning of culture (t_0) in both control and experimental culture system. Moreover, the addition of bPRL significantly (P<0.05) decreased level of GC apoptosis after IVM in comparison with culture system without bPRL (Table 2).

Determination of apoptosis showed beginning of cell death pycnosis which indicate the death of cell nucleus. IVM significantly (P<0.05) increased pycnosis in GC. The proportion of pycnosis in GC after IVM significantly was lower (P<0.05) in bPRL culture in comparison with control culture system as in Table 3.

Table (2): Effect of bPRL on the apoptosis in granulosa cells after IVM

Culture system	Culture time	No.of cells (total)	No.of cells with apoptotic nuclei (n)	% cells with apoptotic nucleus changes
TCM 199 ± 10% FCS	0	9523	2760	29 [a]
	24	10201	4896	48 [b]
TCM 199 ± 10% FCS ± 50 ng /ml bPRL	0	9523	2761	29 [c]
	24	11140	3787	34 [d]

Values followed by the different letters are significantly different at P<0.05 (within column).

Table (3): Effect of bPRL on the level of pycnotic nuclei in granulosa cells after IVM

Culture system	Culture time	No. of cells (total)	No. of cells with pycnotic nucleus (n)	% of cells with pycnotic nuclei
TCM 199 ± 10% FCS	0	9300	1395	15 [a]
	24	8700	3393	39 [c]
TCM 199 ± 10% FCS ± 50 ng/ml bPRL	0	9300	1395	15 [a]
	24	7800	2262	29 [b]

Values followed by the different letters are significantly different at P<0.05 (within column).

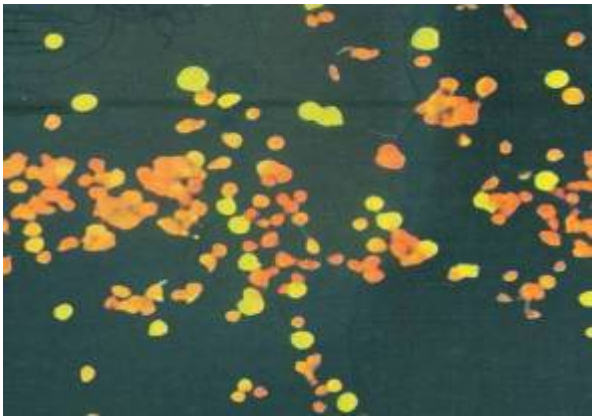


Fig. (1): Apoptosis in GC at the time of recovery (normal cells stained orange with PI and Apoptotic cell nuclei stained with TUNEL green-yellow) X 600

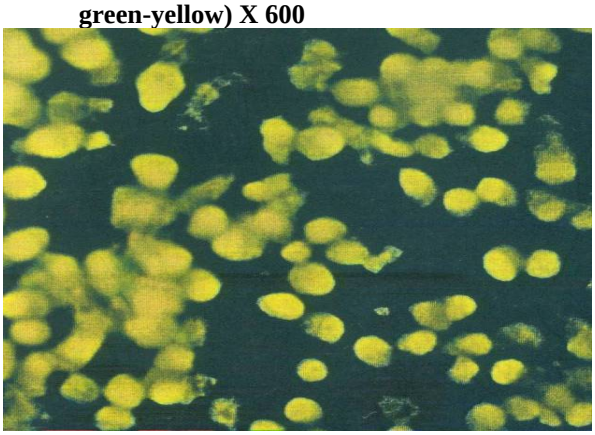


Fig. 2: Apoptosis in GC after 24 hour IVM (normal cells stained orange with PI and Apoptotic cell nuclei stained with TUNEL green-yellow) X1200

Experiment III

To evaluate the status of mitochondria and level of $[Ca^{2+}]_{is}$, only intact oocytes with morphological signs of maturation (cumulus expansion, extrusion of polar body) were used after IVM. Data in table (4) demonstrate the fluorescence intensity of the oocytes prelabeled by vital mitochondria specific probe CMTM Ros and measured by fluorescence intensity at 570-nm was oocytes was significantly (P<0.05) increased in oocytes matured in the presence of bPRL compared with those matured in the absence of bPRL (254.3 nd 119.9 μA, respectively). The fluorescence intensity of oocytes, based on their metabolically active, respiring mitochondria was influenced by culture media. In parallel, there was a slight tendency for higher proportion of oocytes with aggregated mitochondria after IVM with bPRL and GC (Fig. 3).

The level of $[Ca^{2+}]_{is}$ in oocytes as in Table (5), indicated by the fluorescence intensity calcium CTC-complexes/oocytes, was significantly (P<0.05) decreased under the influence of bPRL and GC in compare with bPRL alone (067 AU<1.0 AU)

Table (4): Effect of bPRL on mitochondrial activity(fluorescence intensity per oocyte based on vital labelling of metabolically active mitochondria)in bovine oocytes after IVM with or without GC (n = 82 oocytes)

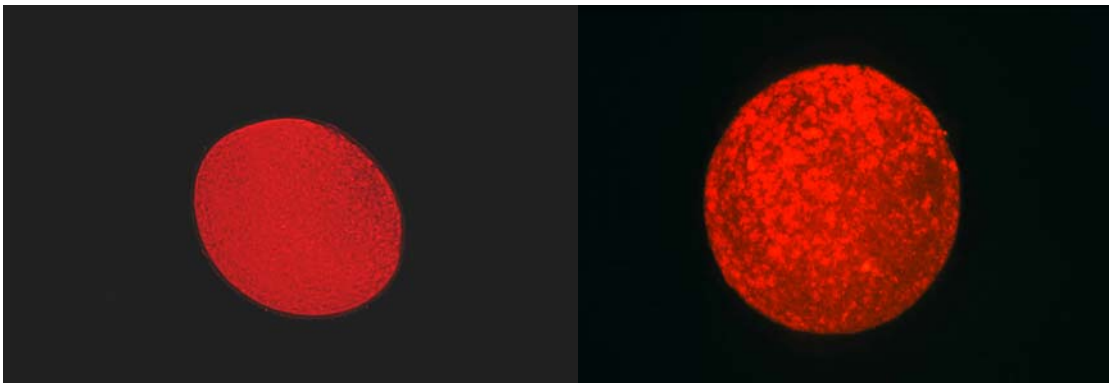
Culture system (n oocytes)	Fluorescence intensity (µA ± SEM)
TCM 199 + 10% FCS + 50 ng/ml bPRL (n=39)	119,9 ± 20,4[a]
TCM 199 + 10% FCS + 50ng/ml bPRL + GC (n=43)	254,3 ± 20,2[b]

Values followed by the different letters are significantly different at P<0.05 (within column).

Table (5): Effect of bPRL on the levels of intracellular stored calcium $[Ca^{2+}]_{is}$ in bovine oocytes after IVM with or without GC (n=78)

Culture system (n oocytes)	Fluorescence intensity of calcium-CTC complexes in oocytes (A.U. ± SEM/oocyte)
TCM 199 + 10% FCS + 50 ng/ml bPRL (n=34)	1,0 ± 0,07[a]
TCM 199 + 10% FCS + 50ng/ml bPRL + GC (n=44)	0,67 ± 0,06 [b]

Values followed by the different letters are significantly different at P<0.05 (within column)



A B

Fig.(4): Visualization of bovine oocytes stained with Mito Tracker M7510 at 250 magnification (A) homogenous non-aggregated mitochondrial distribution after IVM (B) aggregated mitochondrial distribution after IVM

Experiment IV

Analysis of cytogenetic and morphological parameters revealed bPRL had an influence on subsequent embryo quality and bPRL exerts its action through GC. Data concerning the effect of bPRL and GC during IVM on subsequent embryo quality is shown in Table (6). Proportions of intact embryos were significantly (P<0.05) higher in the group of embryos originating from oocytes culture in presence of GC and bPRL in comparison to that culture with bPRL only (32 vs 55, respectively).

Table (6): Effect bPRL on embryos quality after IVF of *in vitro* matured oocytes with or without GC (n=201 embryos)

Culture system	No. embryos	No. degenerated embryos (visual analysis)*	No. degenerated embryos **	No. (%) degenerated embryos total
TCM199 + 10% FCS + 50 ng/ml bPRL	98	27	27	54 (55,1)[a]
TCM 199 + 10% FCS + 50ng/ml bPRL + GC	103	15	18	33 (32) [b]

Values followed by the different letters are significantly different at P<0.05 (within column)..

*Visual analysis – lyses of blastomeres, destructive of shell, block on 2-16 cell stage, vacualization, fragmentation, etc.** Cytological analysis – Embryos with empty blastomeres, bi- or multinuclear blastomeres, with blastomeres including pycnotic chromatin, blastomeres without chromatin were classified as not intact.

4. Discussion:

Prolactin (PRL) has also been shown to exert an effect on mammalian ovary (Doppler, 1994;. Prolactin has many important biological functions in female reproductive system. The role of PRL in the mechanism of meiosis and folliculogenesis regulation is not fully understood. The preovulatory transient rise level of PRL has been seen in blood and follicular fluids of some species (Kuzmina, et al., 1999) which is presumed to be a result of estradiol (Wise et al., 1994). Prolactin is essential for physiological function of corpus luteum: PRL inhibits corpus luteum catabolism and is responsible for keeping up the number of LH and estradiol receptors. PRL is recognized by prolactin receptors which are localized on granulosa cells (Leroy-Martin et al., 1989).

In the investigation, there was a significant increase in fertilization, cleavage rate and morulae, blastocyst formation after addition of both bPRL and granulosa cells in comparison to GC and bPRL alone. Our findings are indicative, the influence of bPRL on developmental competence occurred through GC during oocyte maturation and exerts the promontory effect on bovine oocyte maturation, mainly in cytoplamic maturation and make synchrony between nuclear and cytoplasmic maturation which are criteria for subsequent embryo development. Similar results obtained by Mattioli et al., (1988) after addition of granulosa cells to the culture medium improved cytoplasmic maturation of oocytes which determined by male pronuclear formation. The culture medium providing more physiological environment and addition of bPRL trigger maturation promoting factor (MPF) (Torner et al., 2001) PRL have been shown to enhance developmental competence in mature rabbit (Yoshimura, et al.,1989) and bovine oocytes [Alm et al.,1998; Heleil et al., 2001). Using an in vitro-perfused rabbit ovary model, prolactin also showed to inhibit hCG-induced ovulation in dose-dependent manner by inhibiting activity of hCG plasminogen activator in mature follicles (Yoshimura et al. 1991). For these reasons we realized the effect of PRL on developmental competence of bovine oocytes through influence of PRL on delay of nuclear maturation through substances secreted by granulosa cells and give chance for synchrony of cytoplasmic and nuclear maturation similar to occur in vivo.

Apoptosis is defined a self destruction of cells under physiological control (Ameisen, 2002). Typical features of apoptotic cells include cell shrinkage, translocation of phsopho-serine to the outer cytoplasmic membrane, DNA fragmentation and segmentation of the cell to apoptotic bodies (Ameisen, 2002). Follicular degeneration as well as development is linked to atresia. Follicular atresia occurred via apoptosis and pycnosis (Jolly et al., 1994). Apoptosis can breakdown into four stages. Stimuli, signals, regulators and effectors, before the cell enter into latter stages of regulation, apoptosis is a reversible process (Morita, & Tilly, 1999). Our results indicated the increased apoptosis in GC after IVM, which consider as signs of maturation and differentiation process. Heleil et al., (2001) proved that apoptosis in GC as physiological process increased after IVM, similar to occurred in vivo in preovulatory follicles (Jolly et al., 1994). After addition of PRL to maturation in the present study observed significantly decreased apoptosis in GC after IVM. Our results suggest that PRL protect GC from apoptosis or may affect on the apoptosis before enter later stage and make it reversible. In support our results, a decline follicular fluid PRL concentration in bovine ovarian follicles was associated with increase apoptosis in GC (Lebedeva et al., 1998)

Estimation of apoptosis in GC indicator for early begin cell death while pycnosis indicator for late stage of cell death and follicular atresia (Grimes et al., 1987). Parallel to our results of apoptosis after addition of PRL decreased also number of pycnotic nuclei of GC after IVM (Tarkowski, 1966). PRL interaction with GC, decrease apoptosis and pycnosis level in GC may be due to PRL stimulate substance inhibiting cell death or alteration of Ca^{2+} of GC where Ca^{2+} play a role in cell death. Intracellular Ca^{2+} concentration can trigger apoptosis, PRL stimulate Ca^{2+} entry from intracellular store (Kuzmina et al., 1999) which proved in our results.

Two markers were used for evaluation of cytoplasmic maturation of bovine oocytes co-cultured with granulosa cells; we compared the mitochondrial activity and the content of calcium in the intracellular depots of oocytes matured in the presence or absence of GC and bPRL. The central role of mitochondria in a metabolism and bioenergetics is well defined. It has been shown that the level of mitochondria can be used for assessment of the functional status and quality of oocytes in pig, cattle and horses(Kuzmina et al., 1998; ; Stojkovic, et al., 2001; Torner et al., 2004; Torner et al., 2007). In the present study, the oxidative activity of mitochondrial oocytes matured in the presence of PRL with granulosa cells was significantly increased in comparison to the culture with PRL and without GC. The reason for the increase in fluorescence intensity of labeled mitochondria during maturation is likely an increase of respiratory activity to provide ATP for activation and pre-implantation embryo development. Finding concerning increasing levels of fluorescence intensity of labeled mitochondria during porcine and bovine oocyte maturation (Torner et al., 2004; Tarazona, et al., 2006) positively correlates with results of other

authors (Tarkowski, 1966), who found increased levels of ATP content/oocyte during IVM.

Calcium is an important intracellular messenger, and calcium ions regulate many key intracellular calcium events. It is known that calcium-dependent signaling pathways are involved in the regulation of mammalian oocyte meiotic maturation (Herbert et al., 1997). A relationship between calcium stores and developmental competence has been also described for in vitro matured bovine oocytes (He et al., 1997). Moreover, normal oocytes have low levels of [Ca^{2+}] immediately after removal from the follicles in comparison to oocytes with signs of degeneration (Kuzmina et al., 1991). In the present study we found that supplementation with granulosa cells and PRL during IVM decreased the level of intracellular calcium stores in oocytes after maturation. An increase in mitochondrial activity and calcium exit from intracellular stores led to activation of metabolism in matured oocytes.

Bovine embryos derived in vitro are recognized to have lower quality abnormalities in metabolism and high incidence of chromosome abnormalities (Khurana et al., 2000). In analysis of the morphology and chromatin status of in vitro produced embryos on PRL and granulosa cells decreased the proportion of embryos with morphological and chromatin abnormalities. In comparison with the group where oocytes were matured with PRL and GC, we found higher proportion of degenerated embryos with bi- and multinuclear blastomeres, a nuclear cells, pycnotic nuclei, and abnormal cytokinesis in the control group (maturation with PRL only). Therefore, it is evident that the embryos obtained from in vitro matured oocytes in the presence of PRL and granulosa cells had higher potential for continued development to blastocysts.

In conclusion, the results of the present study clearly demonstrate that PRL supplementation together with follicular cells (granulosa cells) has a beneficial effect on the cytoplasmic quality of bovine oocytes and their subsequent development to the blastocyst stage after IVF. In oocytes matured in the presence of PRL and granulosa cells increased levels of respiratory activity were associated with decreased level of calcium from intracellular stores. Granulosa cells under the influence of PRL may produce a stimulatory substance(s) that promotes the potential of bovine oocytes for development to blastocyst stages. Therefore, possible mechanisms of prolactin mediated effect on developmental competence of bovine oocytes need more research.

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