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Influence of Recombinant Bovine Somatotropin and Prolactin on Mitochondrial Activity in Buffalo Oocytes and Their Development Competence *in vitro*

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Abstract: The present study was conducted to evaluate the effects of recombinant bovine somatotropin (rbST) and bovine prolactin (bPRL) on the nuclear and cytoplasmic maturation of buffalo oocytes and their further developmental competence *in vitro* and evaluation of the chromatin status of produced buffalo embryos after *in vitro* fertilization. Furthermore, analysis of mitochondrial activity using the fluorescence probes mitotracker CMTM Ros orange. Buffalo cumulus-oocyte-complexes (COCs) were incubated in TCM199 containing 10% fetal calf serum (FCS) (control medium, CM). The culture medium of the treatment groups was modified by supplemented of the control medium with 10 ng/ml rbST, 50 ng/ml bPRL with or without 1 x 10^6/ml granulosa cells (GC). No differences were observed in the percentages of occytes reaching metaphase II between the groups. However, the proportion of blastocysts was highest in treatment groups, rbST or bPRL plus GC ($P<0.05$). Cytogenetic analysis of embryos, revealed that the proportion of intact 2-4 cells embryo and intact morulae were significant higher ($P<0.05$) in the groups cultured with rbST or bPRL. The fluorescence intensity of metabolically active mitochondria measured by intensity per oocyte (Em 570) was significantly increased in oocytes matured in the presence of 10 ng/ml rbST or 50 ng/ml bPRL plus GC (346.34, 363.28 vs 142.22 μA; $P<0.05$). In conclusion, the results indicate that rbST, bPRL in interaction with granulosa cells stimulate the oxidative activity of ooplasmic mitochondria. These facts support the hypothesis that somatotropin and prolactin influence the developmental competence of buffalo oocytes during *in vitro* maturation and this effect can be modulated by grannulosa cells.

Key words: Buffalo oocyte · Mitochondria · Embryo · rbST · bPRL

INTRODUCTION

Recently, there has been an increasing interest in the *in vitro* embryo production (IVEP) technologies for faster propagation of superior germplasm in buffaloes, due to the low and inconsistent response to multiple ovulation and embryo transfer [1]. There has been an increasing interest worldwide in the IVEP technology in buffaloes. However, the buffaloes IVEP system has been greatly improved over the years, leading to high bastocyst yields [2] and to the production of offspring [3]. This is still poor and far from being commercially viable.

An important requirement for a successful fertilization is undoubtedly the appropriate maturational status of the oocytes at the time they encounter the sperm. The oocyte maturation process, that invests both nuclear and cytoplasmic compartments, triggered *in vivo* by the gonadotropin surge, which is imperative for the gamete to acquire full developmental competence [4]. Embryo development influenced by events occurring during oocyte maturation. The developmental potential of oocytes is related to many factors, such as the morphology of cumulus-oocyte complexes (COCs), the diameter of follicles and culture system [5]. One important factor in the complex of fertility events is the growth hormone, somatotropin (ST) [6, 7], which influences the regulation of growth and differentiation of various cell types and control of metabolic organ and tissues. Addition of ST during IVM of bovine COCs accelerates nuclear maturation, induces cumulus expansion and promotes subsequent embryonic development [8, 9].

Hormonal supplement to IVM medium are known to be required to promote cytoplasmic maturation of bovine oocytes [10]. Compounds like prolactin have shown to

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enhance developmental competence of *in vitro* matured rabbit [11], human [12] and bovine oocytes [10, 13, 14]. Prolactin has established roles for cellular proliferation and differentiation and has also identified as anti-apoptotic agent [15].

The cytoplasm of the oocyte may play a crucial role in assembling the correct metabolic machinery for production of sufficient energy for cellular function during maturation, cleavage and blastocyst formation [16, 17]. Mitochondria provide energy for the intensive metabolism of oocytes during final maturation [18].

The aims of the present study were to (i) determine the influence of recombinant bovine somatotropin (rbST) and bovine prolactin (bPRL) on the developmental competence of buffaloes oocytes, (ii) determine the influence of rbST and bPRL on the ongoing embryonic quality of oocytes on days 2 and 5 post IVF and (iii) evaluate of the effects of rbST and bPRL on the cytoplasmic quality by measurement of the mitochondrial activity of oocytes after IVM.

MATERIALS AND METHODS

Recovery of Oocytes and Preparation of Granulosa Cells: Ovaries from buffaloes were obtained at a slaughterhouse (Kafrelsheikh, Tanta and Cairo, Egypt) and transported within 2-4 h to the laboratory at 30 to 35°C in a prewarmed thermos container with phosphate buffer saline (PBS).Upon reaching the laboratory, the ovaries were washed in fresh medium (TCM199, Sigma Chemical Co., St. Louis, Mo, USA) and COC were recovered from follicles 2 to 8 mm in diameter by aspiration through 18-guage needle attached to a 5 ml syringe. COC were washed in TCM199 supplemented with 10% heat-treated (56°C, 30 min) FCS (Sigma). All preparations were carried out in the laboratory at 37°C. Prior to culture, COC were evaluated under binocular microscope using morphological criteria. Only oocytes having complete, compact, multilayered cumulus and homogenous ooplasm were used for culture. Before cultivation the oocytes were washed twice in TCM 199 with 10% FCS.

Granulosa cells (GC) were collected from the medium from which COCs were dissected from follicles (2 to 8 mm in diameter) or from follicular fluid of aspirated follicles 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.

Oocyte Maturation: Groups of 15 to 20 buffalo COC were randomly distributed in 50µl drops of culture medium overlaid with mineral oil. The COC were culture for 24 h at 38.5°C (under 5% CO_2 in 100% humidified air) in different treatment groups based on following culture system.

Group 1: Oocytes (n=159) matured in TCM199 (25 mM HEPES with Earle's salt and L-glutamine, Sigma USA) supplemented with 10% heat treated fetal calf serum (FCS; Sigma) and 50 µg/ml gentamycin (Sigma). (Control media, CM).

Group 2: Oocytes (n=170) CM+rbST matured in control media plus 10 ng/ml rbST (Elanco, Greenfield, IN) [8].

Group 3: Oocytes (n=183), CM+bPRL, matured in control media plus 50 ng/ml bPRL (20 IU/mg, institute of Endocrinology, Moscow, Russia) [14].

Group 4: Oocytes (n=172), CM+GC, matured in control media plus 1 x 10^6/ml granulosa cells [19].

Group 5: Oocytes (n=190), CM + GC + rbST, matured in control media in addition of 1 x 10^6 granulosa cells plus 10 ng/ml rbST.

Group 6: Oocytes (n=182) CM + GC+bPRL, matured in control media plus 1 x 10^6 granulosa cells and 50 ng/ml bPRL.

***In vitro* Fertilization (IVF):** After IVM, matured oocytes from different culture systems were fertilized *in vitro* using frozen-thawed buffaloes semen. The frozen semen of buffalo bull, known for high rates of IVF and cleavage was used. A motile sample of sperm was obtained by swim-up separation based on the method of Lonergan *et al*. [20]. 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 ml droplet of fertilization medium (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 100% humidified air.

***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 and

France). Twenty-four hours later after replacement in the culture, the cleaved embryos were transferred to previously prepared buffaloes oviductal epithelial cell (BOEC) monolayer formation were done as described by Nandi *et al*. [21]. After 3 days of culture, 40 µl of the culture medium was replaced with fresh embryo culture medium. On day 7 (day 1 = day of fertilization), the percentage of blastocyst were recorded.

Evaluation of Oocytes and Embryos: Nuclear maturation of the oocytes was evaluated using the cytogenetic method described by Torner *et al*. [10]. 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 a light microscope at x 900 magnification for determination of nuclear status. The procedure to estimate the chromatin status of embryos on day 2 (2- to 4-cell stages) and day 5 (morulae) was the same as described for oocytes [22]; however, before analysis, the embryos were also incubated in 0.1% hyaluronidase for 20 min. Embryos with bi- or multinuclear blastomeres, blastomeres including pycnotic chromatin and blastomeres without chromatin were classified as not intact.

Fluorescence Labeling of Mitochondria and Measurement of Fluorescence Intensity: The *in vitro* matured COCs were processed to evaluate mitochondrial patterns and activities as described by Torner *et al*. [17]. COCs were incubated for 30 min in PBS containing 3% BSA and 200 nM MitoTracker Orange CMTM Ros (Molecular Probes, Oregon, USA) under culture conditions. The cell-permeant probe MitoTracker Orange-fluorescent tetramethylrosamine (M-7510) is readily sequestered only by actively respiring organelles dependent upon their oxidative activity. Then the cumulus cells were removed as described above and the oocytes were washed three times in pre-warmed PBS without BSA. The oocytes were then fixed for 15 min at 37 °C using freshly prepared 2% paraformaldehyde in Hank's balanced salt solution. The probe M-7510 contains a thiol-reactive chloromethyl moiety and can react with accessible thiol groups on peptides and proteins to form an aldehyde-fixable fluorescent conjugate, which is well-retained after cell fixation over a period of six weeks. After fixation the oocytes were washed three times in PBS, mounted on slides under cover slips and stored in the refrigerator prior to fluorescence microscopy evaluation. An epifluorescence microscope (Jenalumar, Carl Zeiss and 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 buffalo oocytes was characterized only by observation (up to 500 x magnifications) of the labeled mitochondria which were oxidative active. The aggregation patterns were mainly classified as fine (small pixels of fluorescence intensity throughout the cytoplasm), or aggregated (larger liner aggregations of fluorescence intensity). The fluorescence intensity (uA) was measured by the 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 data of emission intensity/ oocyte were reduced by compensation for the background fluorescence.

Experimental Design

Three Series of Experiments Were Carried out. First Experiment: COCs were matured *in vitro* in the presence or absence of rbST or bPRL plus supplementation of GC for 24 h (fixed to evaluate maturation capacity). Another group of COCs culture in IVM media as above for 24 h and then processed for IVF to determination cleavage rate after 2 days or blastocyst rate at 7 days post IVF.

Second Experiment: COCs were cultured *in vitro* in presence or absence of rbST or bPRL and IVF, for determination embryo quality at day 2 (2-4 cell stage) and day 5 (morulae stage) after fixation of embryos. Third experiment COCs matured in the presence or absence of rbST or bPRL where oocytes prelabeled by vital mitochondria specific probe CMTM Ros and measured fluorescence emission intensity for evaluation cytoplasmic maturation by measurement of the mitochondrial activity.

Statistical Analysis: 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. Statistical analysis of the fluorescence intensity of mitochondria was conducted using the SAS system for windows (release 8.02). Elementary statistics and frequency tables were calculated with help of the procedures of Means and Freq in SAS/BASE software.

RESULTS

Experiment I: A total of 1056 buffalo compact COCs were used for determination the effect of rbST and bPRL on the developmental competence. In comparison of the proportions of matured oocytes, i.e. metaphase II stage oocytes, cleaved embryos and blastocysts after IVM. The data concerning the effect of rbST and bPRL in buffaloes oocytes maturation in different culture systems is shown in Table 1. There were none significant differences in the percentages of oocytes reaching metaphase II stage in all groups of experiments. As compared with controls, supplementation with rbST, bPRL or granulosa cells (GC) alone lead to increase in development to blastocysts stage, but the highest level of blastocysts was observed after oocyte maturation with rbST plus GC and bPRL supplemented with GC together (P<0.05). The positive effect of rbST and bPRL on developmental competence of buffalo oocytes depend on the presence of granulosa cells during IVM.

Experiment 2: These investigations were done with only two culture system rbST or bPRL supplemented with FCS and 1 x 10^6 granulosa cells/ml. Analyses of cytogenetic and morphological parameters revealed that rbST and bPRL had an influence on subsequent embryo quality. Figure 1: summarizes the data concerning the effect of rbST and bPRL during subsequent embryo quality. The proportions of intact 2 to 4 cell embryos on day 2 and proportion of intact morulae on day 5 were higher in the group of embryos originated from oocytes cultured with rbST and bPRL (P<0.05).

Experiment 3: As in second experiment, TCM 199 with 10% FCS and 1 x 10^6 granulosa cells/ml for maturation of the oocytes in the presence or absence of 10 ng/ml rbST or 50 ng/ml bPRL was used. For evaluation of the mitochondrial status, only intact oocytes with morphological signs of maturation (Cumulus expansion, extrusion of polar body) were used after IVM. The data in Table 2 demonstrates the fluorescence intensity of

Table 1: Effect of rbST, bPRL and granulosa cells (GC) on the developmental competence of buffalo oocytes matured in TCM199 with FCS (CM) (n=1056)

Culture system	Matured-M II% (N)	Cleavage% (N)	Developed blastocyst% (N)
Control media (CM)	74.1(63/85)	36.5(27/74)[a]	12.2(9/74)[a]
CM + rbST	79.6(78/98)	48.6(35/72)[a]	27.8(20/72)[b]
CM + bPRL	81.3(83/102)	49.4(40/81)[a]	29.6(24/81)[b]
CM + GC	77.0(74/96)	46.0(35/76)[a]	26.3(20/76)[b]
CM + GC + rbST	82.9(87/105)	67.1(57/85)[b]	42.4(36/85)[c]
CM + GC + bPRL	84.2(75/89)	71.0(66/93)[b]	48.4(45/93)[c]

a:b, a:c, b:c P<0.05 (within columns)

Table 2: Fluorescence intensity/oocyte and mitochondrial aggregation based on vital labeling of metabolic active mitochondria in buffalo oocytes after IVM

Treatment group	Fluorescence intensity/oocyte (µA±SEM)	Oocyte with mitochondria in aggregation% (N)
CM + GC	142 ±22.8[a]	16.7 (6/36)[a]
CM + GC + rbST	346±34.6[b]	37.0 (10/27)[b]
CM + GC + bPRL	363±28.5[b]	43.6 (14/32)[b]

a:b P<0.05 (within columns)

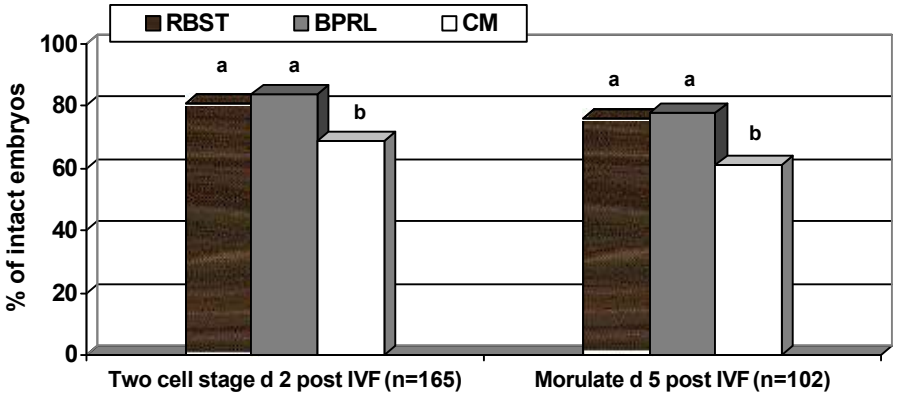

Fig. 1: Embryo quality on day 2 and 5 after IVF *in vitro* matured in TCM199 with FCS (CM) + GC with or without rbST, bPRL (n=267 embryos) a:b (P<0.05)

the oocytes prelabeled by vital mitochondrial specific probe CMTM Ros and measured by fluorescence intensity at 570 nm emission /oocyte was significantly increased in presence of 10 ng/ml rbST, 50 ng/ml bPRL compared with those matured in absence of rbST or bPRL (346.3, 363.2 and 142.2 μA, respectively; $P<0.05$). In parallel, there was a significant increase ($P<0.05$) in the proportion of oocytes with aggregated mitochondria after IVM with rbST or bPRL.

DISCUSSION

This study confirmed that the supplementation with rbST and bPRL in presence of granulosa cells improve the developmental capacity of buffaloes oocytes which reflected by the enhanced blastocyst formation. The increase of developmental competence after addition of rbST or bPRL to IVM media points to the effect of rbST and bPRL on kinetics of meiosis and cytoplasmic elements indeed for successful IVEP of buffalo oocytes.

It has been shown that growth hormones are involved in the processes of sexual differentiation, pubertal maturation, gonadal steroidgenesis, gametogenesis and ovulation [6]. Boland *et al*. [23] found that effects of rbST are related to beneficial effect of IGF-1 on preovulatory follicle and on oocytes with a possible positive effect on subsequent CL function. Treatment superovulated donor cows with growth hormone (GH) at the time of insemination decreases the number of unfertilized oocytes and increases the percentages of transferable embryos. As shown in an *in vitro* model, both GH and IGF-1 stimulate embryonic development in cattle and the effects of GH may likely involve IGF-1 independent mechanism [24]. The stimulatory effect or rbST during IVM on the development of buffaloes oocytes was also confirmed in this study. Moreover, this effect is amplified in the presence of granulosa cells.

Prolactin tends to have the same action of GH, regulates many gonadal and plays an important peripheral role in female reproductive function. In human beings, PRL can support oocyte developmental competence during maturation [25]. PRL is essential for the physiological function of CL: PRL inhibits CL catabolism and PRL is responsible for keeping up the number of LH and oestradiol receptors [26]. PRL is recognized by receptors which are localized on granulosa cells [26]. The results of Yoshimura *et al*. [11] indicated that addition of PRL to IVM enhances the developmental competence of *in vitro* matured rabbit oocytes to morulae and blastocyst stage in a dose dependent manner. PRL induces mobilization of Ca^{++} (in bovine oocytes) from intracellular stores in such cells are responsive to mitogenic activity [27] as well as the mitogenic activity of this hormone on bovine granulosa cells [28]. In contrast, many investigators found evidence of detrimental effects of high bPRL level on oocyte maturation by inhibiting biosynthesis of estrogens and also by stimulating granulosa cells to secretion substance that inhibit oocyte maturation [29]. Using an *in vitro* perfused rabbit ovary model, PRL induced ovulation in a dose dependent manner by inhibiting activity of HCG-stimulated plasminogen activator in mature follicles [12].

Oocyte grows with follicles multicellular units comprising the oocyte and its surrounding granulosa cells. The supplementation IVM media with GC improved cytoplasmic maturation of oocytes, it is likely that GC could be secreting some meiosis promoting factor within culture media [19]. It is hypothesized that such secreted factors could remain active within GC cultures condition medium under cell free condition which could in turn lead to enhancement of oocyte maturation [30]. The results of the present study confirmed that addition of GC to culture media improved developmental competence of buffalo oocytes. Furthermore, supplementation of IVM media with rbST or bPRL with GC showed a significant ($P<0.05$) increase of developmental competence in comparison with IVM supplemented either rbST or bPRL alone without GC. These results indicated a possible influence of buffaloes granulosa cells on rbST and bPRL related effects on cytoplasmic maturation and developmental competence *in vitro*.

Bovine embryo derived *in vitro* is recognized as being of lower quality than those obtained following superovulation, artificial insemination and non-surgical recovery [31]. *In vitro* produced embryos have abnormalities in metabolism and a high incidence of chromosome abnormalities [32]. In analysis of the morphology and chromatin status of *in vitro* produced embryos on day 2 and 5, rbST and bPRL decreased the proportion of embryos abnormalities. In comparison with the experimental group, the present results revealed that a higher proportion of degenerated embryos with bi-and multinuclear blastomeres, a nuclear cells, pycnotic nuclei and abnormal cytokinesis in the control group. Therefore, it is evident that embryo obtained from *in vitro* matured oocytes in the presence of rbST, bPRL and granulosa cells had a higher potential for continued development to blastocyst.

The mitochondrial activity which can be used for assessment of the functional status and quality of oocytes in pig, cattle, horses [17, 18, 22, 33]. In the present study, the oxidative activity of mitochondria in oocytes matured in the presence of rbST, bPRL was significantly increased in comparison with control group. 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 preimplantation embryo development. Findings concerning increasing levels of fluorescence intensity of labeled mitochondria during porcine and bovine oocyte maturation [17, 34] positively correlate with the results of other authors [18, 35) who found increased levels of ATP content/oocyte during IVM.

In conclusion, the results of the present study clearly demonstrate that rbST or bPRL supplementation together with follicular somatic (granulosa cells) has a beneficial effect on cytoplasmic quality of buffaloes oocytes and their subsequent development to blastocyst stage after IVF. Granulosa cells under the influence of somatotropin or prolactin may produce a stimulatory substance (s) that promotes the potential of buffalo oocytes for development to the blastocyst stages.

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