The effect of prolactin and bovine follicular fluid on the *in vitro* maturation and subsequent development of immature buffalo (*Bubalus bubalis*) oocytes

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Abstract: Buffalo oocytes with compact cumulus cells were collected from slaughterhouse ovaries and cultured in TCM 199 supplemented with 5% estrus cow serum (ECS) + 0.1 μg/mL follicle stimulating hormone (FSH), prolactin (PRL 0, 0.1, 1.0, 10 μg/mL, Experiment 1), bovine follicular fluid (BFF) (0% BFF + 1.0 μg/mL PRL, 5% BFF, 5% BFF + 1.0 μg/mL PRL, Experiment 2), incubated at 38.5 ℃ in φ = 5% CO_2 in humidified air. After 24 to 26 hours of maturation, IVF was done with swim-up separated frozen-thawed buffalo spermatozoa at 1×10^6 mL^{-1} in modified Tyrodes medium (TALP). At 24 to 26 hours post insemination, the oocytes were co-cultured with granulosa cell monolayer in droplets containing culture medium. The proportion of cleaved oocytes that developed to blastocyst stage within 9 days after commencing co-culture with granulosa cell monolayer was then evaluated. In experiment 1, the proportion of cleaved oocytes that developed to blastocyst stage was higher (12.8%) at 1.0 μg/mL PRL though not significantly different from the control (9.1%). In experiment 2, addition of 5% BFF to the maturation medium had significant increase in the cleavage rate of oocytes compared to the control (30.7% vs. 21.7%, $P < 0.05$), but did not influence the proportion of cleaved oocytes that developed into blastocysts; addition of 5% BFF + 1.0 μg/mL PRL to the maturation medium had a cleavage rate of 38.1%, with 14.0% of the cleaved oocytes developing to blastocysts ($P < 0.05$). In conclusion, the results indicate that the addition of appropriate amounts of prolactin and bovine follicular fluid to the maturation medium will enhance the maturation of immature buffalo oocytes with their subsequent development to the blastocyst stage.

Key words: buffalo; *in vitro* maturation; bovine follicular fluid; prolactin; blastocyst
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催乳素和牛卵泡液对胚胎体外成熟及未成熟的牛卵母细胞的影响

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摘要:对催乳素和牛卵泡液在水牛卵母细胞体外成熟中的作用进行了探讨. 来自屠宰场水牛卵巢的卵母细胞和卵丘细胞复合体,在含体积分数为5% CO_2 的培养箱中培养24~26 h,然后通过体外受精测定其受精和胚胎发育能力. 实验1在成熟液中添加1.0 μg/mL 催乳素(PRL),卵母细胞的囊胚发育比例(12.8%)比对照组(9.1%)高但不显著. 实验2添加5%牛卵泡液(BFF),卵母细胞卵裂的比例明显高于对照组,但囊胚形成率则几乎一样;添加1.0 μg/mL PRL和5% BFF组卵母细胞卵裂的比例为38.1%,而发育成囊胚阶段的卵母细胞的比例为14%. 试验结果表明:添加适宜浓度的BFF和PRL能促进未成熟的水牛卵母细胞体外成熟后的胚胎发育能力.

关键词:水牛; 体外成熟; 牛卵泡液; 催乳素; 胚泡

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Buffalo cows are usually slaughtered when their fertility and productivity are compromised or when the animals are aged, and their follicular reserve is reduced. High incidence of follicular atresia 82%[1] or 92%[2] as observed in slaughterhouse ovaries made the mean recovery of good quality oocytes per ovary highly reduced: 0.4[2,3], 0.9[4], 1.76[5], and 2.4[6]. Gonadotrophins (FSH and LH) and ovarian steroid hormones are known to be involved in the control of follicular atresia[7,8]. To date, it has been shown that PRL is the third pituitary hormone serving gonadotrohic function in mammals[8,9]. However, its role in the regulation of ovarian folliculogenesis and in particular, the relationship between PRL and the processes connected with atresia of ovarian follicles, are poorly understood[10].

Blastocyst stage embryos have been successfully obtained using buffalo follicular fluid in the IVM of buffalo oocytes[11]. Comparative studies on the efficacies of buffalo follicular fluid have been inconsistent, probably because of high variability that exists within and between batches of ovaries. Bovine follicular fluid (BFF) aspirated from large follicles has been shown to have beneficial effect on the acquirement of developmental competence of oocytes matured *in vitro*[13]. Follicular fluid has also been proved to have positive effect on *in vitro* fertilization and embryonic development when added to *in vitro* maturation medium in the pig[13], and human[14]. Nuclear and cytoplasmic maturation is an important process for complete fertilization and subsequent embryo development[15]. It is known that nuclear maturation is regulated by the activity of a cytoplasmic maturation-promoting factor[16] (MPF). The MPF is activated at the onset of germinal vesicle breakdown and chromosome condensation, with peak activity at metaphase I and metaphase II. Sperm penetration lowers MPF activity and promotes entry into anaphase II[17]. Modification of cumulus cell cytoskeleton might be caused by onset, progression, and completion of oocyte maturation[18]. Therefore, bovine follicular fluid (BFF) might alter the expression of MPF in oocytes through cumulus cell communication after LH stimulation. The aim of the present study was to investigate the effect of bovine follicular fluid (BFF) and prolactin (PRL) on *in vitro* maturation and subsequent development to blastocyst stage of immature buffalo oocytes.

1 Materials and methods

1.1 *In vitro* maturation

Buffalo ovaries were obtained from local slaughterhouses excised after slaughter and stored in thermos flasks containing normal saline solution at 25 ~ 30 ℃. The ovaries were transported to the laboratory within three to four hours after slaughter. Oocytes were aspirated from follicles of 2 to 6 mm in diameter using a 5 or 10 mL disposable syringe with an 18-guage needle. Oocytes possessing compact enclosed cumulus cells were selected for maturation.

The basic maturation medium was TCM 199 + 5% ECS + 0.1 μg/mL FSH supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin. The pH of the medium was adjusted to 7.3 - 7.4. The medium was sterilized by passing through a 0.22 μm diameter millipore filter. The washed oocytes were cultured in glass dishes (about 50 oocytes/dish) containing 2 mL of maturation medium, and incubated for 24 ~ 26 hours under 38.5 ℃ with $\varphi = 5\%$ CO_2 in humidified air.

1.2 *In vitro* fertilization of oocytes

Frozen buffalo semen was used throughout in this study. Active motile sperm were separated by swim-up process. The medium for swim-up was modified Ca^{2+} free tyrodes medium with a pH of 7.4, sterilized by passing through a 0.22 μm millipore filter. The medium was incubated for at least 15 minutes prior to use. The frozen buffalo semen in 0.5 mL straws were thawed at 37 ℃ in a water bath for 1 minute. The thawed semen was then layered under 1 mL of Tyrodes medium in conical tubes, and incubated for at least 15 minutes. At the end of the incubation period, the top 0.7 - 0.8 mL of medium was collected into centrifuge tubes. The swim-up separated spermatozoa were washed at (500 g for 5 minutes), after which most of the supernatant discarded and the remainder containing highly motile sperm used for insemination.

The medium used for IVF was modified Tyrodes medium (TALP) containing 80 μg/mL heparin + 0.6% BSA with 2.5 mmol/L caffeine and PHE supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin. The pH of the medium was adjusted to 7.8 and sterilized by filtering through a 0.22 μm diameter millipore filter.

Droplets (25 – 50 μL) of the fertilization medium were prepared in fertilization dishes (50 mm plastic dishes) and covered by sterile mineral oil and then incubated. After 24 – 26 hours of culture in maturation medium, oocytes were prepared for fertilization by partial denudation (mechanical stripping with a micropipette) of the expanded surrounding cumulus cells and then washed in wash medium. Only morphologically normal oocytes were selected for IVF. About 10 to 20 matured oocytes were placed into the droplets under mineral oil. Thereafter the oocytes were inseminated with the swim-up separated sperm (1×10^6) and incubated for 24 – 26 hours.

1.3 *In vitro* culture of early embryos

The medium used for *in vitro* culture of early embryos in this study was TCM-199 + 5% ECS supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin. The pH of the medium was adjusted to 7.3 – 7.4 before filtering through a 0.22 μm diameter millipore filter. After 24 to 26 hours, the inseminated oocytes were co-cultured in droplets containing granulosa cell monolayer. Embryo development was checked at 24-hour intervals and at these times 60% – 70% volume of the culture medium was replaced with fresh culture medium.

1.4 Experimental design

Experiment 1: this experiment was designed to determine the effect of prolactin (PRL) concentration on the IVM of buffalo oocytes. Buffalo oocytes from each batch were randomly allocated into culture in each of the four treatments designated by the concentration of PRL in the basic maturation medium (0, 0.1, 1.0, 10 μg/mL).

Experiment 2: this experiment was designed to determine the effect of bovine follicular fluid (BFF) in the maturation medium on IVM of buffalo oocytes. Buffalo oocytes in each batch were randomly allocated into maturation medium with (0% BFF + 1.0 μg/ml PRL, 5% BFF, 5% BFF + 1.0 μg/mL PRL).

1.5 Statistical analysis

The present study evaluated the IVM of buffalo oocytes by the proportion of cleaved eggs and subsequent development to blastocyst (BL) stage in each experiment within a 9-day period after commencement of co-culture with granulosa cell monolayer. The difference between treatments within each experiment was analyzed by Chi Square.

2 Results

2.1 Experiment 1

The percentage of oocytes that cleaved and developed subsequently to blastocyst stage after IVF and IVC are shown in table 1. The cleavage rate of oocytes in the PRL free group (control group) was significantly lower compared with the treatment group containing PRL at 10 μg/mL ($P < 0.05$). On the other hand, the frequency of cleaved oocytes developing to blastocyst stage did not show any significant difference between the control and the other treatment groups; however, the proportion of blastocysts, which developed at 1.0 μg/mL PRL, was significantly higher than at 10 μg/mL PRL concentration ($P < 0.05$).

Tab. 1 The effect of PRL concentration added to maturation medium on cleavage and subsequent development to blastocyst stage of buffalo oocytes [1)]

ρ(PRL)/ (μg·mL^{-1})	oocytes insem.	No. of cleavage	cleavage rate/%	No. of blastocyst	blastocyst rate/%
0.0	162	33	20.4b	15	9.1ab
0.1	192	51	26.6ab	20	10.4ab
1.0	117	35	29.9ab	15	12.8b
10.0	130	41	31.5a	6	4.6a

1) Within a column, values with different superscripts are significantly different ($P < 0.05$)

2.2 Experiment 2

As shown in table 2, treatment with 5% BFF had significant increase in the cleavage rate of oocytes compared to the control (30.7% vs. 21.7%, $P < 0.05$), but did not influence the proportion of cleaved oocytes that developed into blastocysts. 5% BFF + 1.0 μg/mL PRL had a significant improvement on cleavage and blastocyst yield compared to the control.

Tab. 2 Effect of 5% BFF added to maturation medium on cleavage and subsequent development to blastocyst stage of buffalo oocytes [1)]

treatment	oocytes insem.	No. of cleavage	cleavage rate/%	No. of blastocyst	blastocyst rate/%
0% BFF + 1.0 μg/mL PRL	152	33	21.7a	14	9.25ab
5% BFF	241	74	30.7b	23	9.50ab
5% BFF + 1.0 μg/mL PRL	118	45	38.1c	16	14.00c

1) Within a column, values with different superscripts are significantly different ($P < 0.05$)

3 Discussion

The dominant feature of oocyte maturation is that the female gamete changes from a developmentally incompetent cell to one with the capacity to direct and support the events of fertilization and early embryonic development. The judgment about the competence of oocytes cannot be made until a relatively later stage in embryogenesis, because abnormalities during maturation may be expressed at several stages during development, the most common lesions being observed at fertilization, around compaction, or at blastulation. Accordingly, the present study evaluated the IVM and IVF of buffalo oocytes by the proportion of eggs cleaved and which developed to the blastocyst stage. In our laboratory, the main area of research is on *in vitro* embryo production (IVEP) of cattle and swine. With the growing attention to augment the reproduction of the buffalo in southern China, we desired to use media conditions already established for cattle with some modifications for the production of buffalo embryos simultaneously with that of cattle.

The medium for IVM in our laboratory was TCM 199 supplemented with 5% ECS + 1.0 μg/mL FSH. The results suggested that ECS just as buffalo estrus serum could support the *in vitro* maturation and subsequent development of buffalo oocytes to blastocyst stage. The supplementation of TCM 199 with estrus cow serum (ECS) proved beneficial in cleavage and blastocyst yield when compared with results obtained earlier using buffalo estrus serum[19]. The use of serum introduces proteins and growth factors and may prevent zona hardening, thereby improving the ability of the oocytes to be fertilized. It is absolutely required for cumulus expansion and oocyte maturation and for attainment of normal embryonic development in water buffalo.

According to this experiment, the addition of 1.0 μg/mL PRL to the maturation medium had some beneficial effect as shown by a slight improvement in embryonic development of buffalo oocytes, however, the results also indicate that high levels of prolactin is detrimental or can adversely affect development of buffalo oocytes to blastocyst stage. Elevated PRL levels have been found to act directly on the ovary to suppress follicular development as well as inhibit gonadotropin secretion, and have been reported to assist rabbit oocytes in acquiring their developmental competence[19]. In normal preovulatory women, transient nocturnal hyperprolactinema has been confirmed during the preovulatory phase. Moreover, the clinical data regarding IVF of human oocytes have demonstrated that PRL concentrations in follicular fluids are significantly higher in matured, fertilized ova and in fertilized ova associated with a successful pregnancy than in unfertilized ova[20].

The addition of 5% BFF and 1.0 μg/mL PRL to the maturation medium resulted into a slight improvement in blastocyst yield. Some authors have used 20% buffalo follicular fluid (BuFF) in replacement of hormones and serum additives, obtaining comparable blastocysts rates[11]. Follicular fluid generally contains gonadotrophins, estradiol, progesterone, transforming growth factor-β (TGF-β), and the peptide inhibin. Several growth factors, such as insulin-like growth factor-α (IGF-α), insulin like growth factor-2 (IGF-2), EGF, and transforming growth factor-α (TGF-α) have also been detected in follicular fluids of other species[17]. In fact, recent studies have shown that several ovarian-derived growth factors play an important role in oocyte maturation and post-fertilization development, acting as local modulators of gonadotrophin action on mammalian oocytes. Therefore, BFF, which can be easily collected, can be used as an effective media supplement in the IVM of buffalo oocytes.

The culture conditions in the present study resulted in the yield of blastocyst comparable to other reports. ECS and BFF can be used as convenient alternative media supplement for estrus buffalo serum and BuFF in the IVEP of buffalo.

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