Effect of gonadotrophins, oestradiol and insulin on cumulus expansion of Nili Ravi buffalo oocytes

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Abstract: The objective of the present study was to investigate the cumulus expansions of Nili Ravi buffalo oocytes during cultured in TCM -199 supplemented with 2 µg/ml oestradiol (E_2), 0.05 IU/ml recombinant human follicle stimulating hormone (rhFSH), 2IU/ml human chorionic gonadotrophin (hCG), and 0.12 IU/ml insulin (I). The cumulus oocytes complexes (COCs) were collected from 2-8mm follicles from local abattoir ovaries. Supplementation of medium with single hormones showed significant ($P<0.0001$) increase in mean diameter of COCs with rhFSH except E_2, hCG and insulin after 24 hours compared to the increase in the mean diameter of COCs matured in TCM-199 without any hormonal supplementation. With rhFSH even at 8th hour, significant increase ($P<0.001$) in cumulus expansion was observed. In combination of hormones the significant ($P<0.0001$) cumulus expansion was achieved in E_2+rhFSH treatment group. The non significant ($P>0.05$) cumulus expansion was observed in treatment groups viz. E_2+hCG, E_2+Insulin, rhFSH+hCG, rhFSH+Insulin, hCG+Insulin, E_2+rhFSH+hCG and E_2+rhFSH+hCG+Insulin after 24 hours. In conclusion, supplementation of rhFSH alone and in combination with E_2in TCM-199 has highly significant effect on cumulus expansion.

Keywords: *In vitro*, buffalo, oocytes, gonadotrophins, insulin.

INTRODUCTION

In vitro production of buffalo embryos for research and commercial purpose has been an increasing interest (Mehmood *et al.,* 2011). Because of poor superovulatory response the progress in the use of embryo transfer in the buffalo is very slow.The recovery of immature oocytes followed by *in vitro* maturation and fertilization is an attractive alternative to *in vitro* fertilization (Khalili *et al.,* 2013). The culturing and maturing oocytes that have been harvested at an immature stateare defined as *in vitro* maturation of oocytes (Smith, 2001).

As a result of the preovulatory surge of gonadotropins, in mammalian ovarian follicles the cumulus cells undergo a process of expansion while the residing oocytes undergo a process of resumption and complete meiotic maturation (Sutovsky *et al.,* 1994; Sutovsky *et al.,* 1995; Prchazka *et al.,* 2000). During ovulation the expansion of cumulus cells maintains viability of the ovulated oocytes within the oviduct (Hess *et al.,* 1999). The cumulus expansion is responsible for the creation of the proper micro environment for sperm motility and activation (Tesarik *et al.,* 1988; Chen *et al.,* 1993). In general, cumulus expansion and oocyte maturation is regulated by follicle-stimulating (FSH) hormone and (LH) hormone (Lee *et al.,* 2007). To understand the mechanisms that regulate expansion of cumulus will thus lead to improvement of conditions for mammalian oocytes to be matured and fertilized *in vitro* (Prchazka *et al.,* 2003). FSH can induce *in vitro* cumulus expansion in mouse, pig, rabbit and cattle (Eppig, 1979; Salustri *et al.,* 1992).

The 10µg/ml lectin concentrations showed a significant effect on cumulus expansion in buffalo oocytes (Pandey *et al.,* 2009). For cytoplasmic maturation optimal cumulus expansion is very essential (Testart *et al.,* 1983; Chen *et al.,* 1993). The incidence of oocytes penetration is increased by cumulus expansion in bovine (Ball *et al.,* 1983).

The steroids in follicular fluid may play a role in in-vitro maturation of oocytes (Anisworth *et al.,* 1980). Oocytes are been kept in meiotic arrest by the involvement of estradiol (E_2) and other steroids (McGaughey, 1977; Richter and MeGaughey, 1979; Smith and Tenney, 1980; Racowsky and McGaughey, 1982; Kaji *et al.,* 1987; Barrett *et al.,* 1996; Mingoti *et al.,*1995). The insulin (I) and insulin-like growth factor-I (IGF-I) proliferate the granulose cells and stimulate the progesterone production (Gong *et al.,* 1993; Spicer *et al.,* 1993). Therefore, it is expected that insulin and the growth factor IGF-I have some beneficial effects on bovine oocytes cultured *in vitro*. The cumulus expansion and oocyte fertilizability during in-vitro conditions can be improved by supplementation of maturation media with insulin (Lorenzo *et al.,* 1994; Webb *et al.,* 1996), but it was reported in some other studies that the supplementation of insulin in culture medium did not improve the fertilization rate or morula formation significantly (Stubbings *et al.,* 1990).

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The present study was designed to investigate the effects of supplementation of the maturation media with Oestardiol (E_2), recombinant human follicle stimulating hormone (rhFSH), human chorionic gonadotrophin (hCG) and insulin (I) alone and in various combinations on cumulus expansion of Nili Ravi buffalo oocytes during cultured *in vitro*.

MATERIALS AND METHODS

Preparation of stock solutions

Transport saline (0.9%)

0.9 % saline (9 g NaCl per Liter) was prepared in double distilled water and autoclave. It was label as "sterile 0.9% saline), date was made and stored at 4°C.

Oocytes collection medium

TCM-199 powder (without phenol red and glutamine) was dissolved for 10 liter and 3.50 g NaHCO3 in 9 liter double distal water H2O. pH was adjusted to 7.2-7.4 and volume was brought to10 liter. 400 ml medium was sterile-filter into 500 ml bottles and kept at 4°C.

On night before use following were added; 1 aliquot of bovine steer serum + hepes, 1 aliquot of glutamine (4ml), 0.4 ml of Penicillin G (100IU/ml)and 0.04g of streptomycin sulphate (100 µg/ml).

After supplementation the oocytes collection medium was used within a week.

Oocytes maturation medium

4.45 ml aliquots of TCM-199 were prepared and store at 4°C until use. On night before following were added to an aliquot of TCM-199; 500µl of bovine serum albumen, 50µl of gentamicin.

Following hormones were added according to the experimental design: 10µl of Oestradiol (2 µg/ml), 5µl of recombinant follicle stimulating hormone (rhFSH), 10µl of human chorionic gonadotrophin (hCG), 6µl of beef insulin (0.12IU/ml). The supplemented oocytes maturation medium was used within a week.

All the solutions were placed in a Styrofoam rack by labeling with stock number, solution name, volume and date.

Collection of ovaries

Approximately 560 buffalo ovaries were collected from slaughterhouse. Ovaries were removed from the reproductive tract of buffaloes immediately after internal organ were extracted from the carcass and the ovaries were placed into one of the saline container. The ovaries were transported to the lab within two hours of slaughtering at 37°C. Upon return to the lab the ovaries were washed by massaging several times with the pre warmed 0.9 % saline (W/V) containing 10 IU penicillin/ ml and 10µg streptomycin (Zafa Pharmaceuticals) until majority of the blood had been washed away from the ovaries.

Recovery of cumulus oocyte complexes

To collect the cumulus oocytes complexes (COCs) the follicles (2-8 mm diameter) were aspirated in 5 ml syringe by usinga needle of16 gauge into a beaker containing OCM with supplements. All the contents of follicle were aspirated. The recovered follicular fluid was allowed to stand for about 20 minutes at 30°C in phosphate buffer saline (PBS) containing 25 IU heparin /ml (Leo Pharma) and 50 mg bovine serum albumen (BSA) / ml (Sigma).

The supernatant was removed and the resulting pellet was washed in PBS. Finally the regimented pellet was collected and phase contrast inverted microscope was used for examination. The compact COCs with 3-12 cell layers were selected and washed in maturation medium. The COCS were cultured in 50µl TCM-199 supplemented with sodium pyruvate (0.23m mol /lit), BSA (4.0mg/ml; Sigma), Oestradiol (1mg/100ml; Merck) and rhFSH (0.05 IU/ml; Organon). The droplets of maturation medium were covered with mineral oil. The COCs were incubated in CO_2 incubator (5%) at 38.5°C for 24 hours.

Experimental design

The following experiments were conducted to examine the effect of E_2, rhFSH, hCG and Insulin alone or in various combinations on cumulus expansion of COCs.

- The COCs cultured in TCM199 supplemented with sodium pyruvate, BSA, gentamicin and without any supplementation of E_2, rhFSH, hCG and Insulin served as control.
- The COCs were matured *in vitro* in TCM199 + 2µg/ml E_2.
- The cumulus expansion of COCs was studied in supplemented TCM199 with 0.05 IU/ ml rhFSH.
- The COCs were matured *in vitro* in supplemented TCM199 with 2 IU /ml hCG.
- To study the effect of 0.12 IU/ml of beef insulin on cumulus expansion, the COCs were cultured in supplementedTCM-199.
- The COCs were cultured *in vitro* in supplemented TCM199 in the presence ofE_2 and rhFSH.
- The COCs cultured in TCM-199 in the presence of E_2 and hCG.
- The COCs were cultured *in vitro* in supplemented TCM199 with E_2 and beef insulin.
- The COCs were cultured *in vitro* in supplemented TCM199 in the presence of rhFSH and hCG.
- To study the effect of rhFSH and beef insulin on cumulus expansion the COCs were cultured in the supplemented medium M199.
- Incubation in medium TCM199 supplemented with hCG and beef insulin was evaluated.

- To investigate the effect of E_2, rhFSH and hCG on cumulus expansion, COCs were cultured in the medium TCM-199.
- The effect of oestradiol, rhFSH, hCG and beef insulin on COCs under defined condition in TCM-199.

Assessment of cumulus expansion

The cumulus expansion was observed at the time interval of 8, 16 and 24 hours of incubation under inverted phase contrast microscope (Nikon). Assessment of cumulus expansion was done by the measurement of the diameter of the COCs by using a calibrated stage micrometer in two perpendicular directions.

STATISTICAL ANALYSIS

The increase in the mean diameter of cumulus expansion was compared among control and different treatment groups by applying analysis of variance. Graph Pad Prism 5 package was used for statistical analysis. Difference were considered significant when $P<0.05$.

RESULTS

The COCs with the presence of multi layers (from 3-12 layers) were matured and observed after the time period of 8, 16 and 24 hours. In control group the COCs with an initial mean diameter of 202.5±8.32 µm were cultured. Expansion of COCs in absence of any hormone showed a smalls increase in the diameter at 8 (205.75±8.66 µm) and 16 (208.25±5.89) hours of incubation but after 24 hours the final diameter of COCs reached to 216.75±9.10 µm. The total increase in the mean diameter of COCs in control group was 24.25±7.32 µm after 24 hours of maturation period as shown in Table.

Effect of oestradiol on cumulus expansion

The COCs having mean diameter of233.36±6.68 µm were cultured in TCM199which contained 2µg/ml E_2alone. There was a gradual cumulus expansion observed at 8, 16 and 24 hours of incubation, which was 257.66±9.12, 267.166±9.95 and 276.16±10.2 µm respectively. The supplementation of oestradiol in maturation medium resulted in not significant increase ($P>0.05$) increase of 42.79±8.82 µm in mean diameter of COCs after 24 hours of culture.

Effect of rhFSH on cumulus expansion

The effect of 0.05 IU/ml rhFSH alone on cumulus expansion was studied in TCM-199. The addition of rhFSH stimulated the cumulus expansion. The 202.08±3.28 µm mean diameter of COCs become 223.33±3.82 µm at 8 hours, 276.66±5.80 µm at 16 hours and 352.08±11.5 µm at 24 hours. The stimulatory effect of rhFSH on cumulus expansion of COCs increased the total mean diameter by 150±11.20 µm after 24 hours. This increase was highly significant ($P<0.0001$) compared to control group.

Effect of hCG on cumulus expansion

The COCs selected for maturation in this group was having a mean diameter of 244.25±8.53 µm. Addition of hCG alone showed a very small effect on cumulus expansion of COCs. The mean diameter of COCs observed at 8, 16 and 24 hours of culturing was 245.75±5.12, 250.5±4.07 and 271.75±7.3 µm respectively. The total increase in mean diameter of COCs was 27.50±8.31 µm at the end of culture period of 24 hours, which was not significant ($P > 0.05$) compare to the cumulus expansion in control group.

Effect of insulin on cumulus expansion

The culturing of COCs in medium TCM 199 supplemented with beef insulin (0.12IU/ml) was shown to have no effect on the cumulus expansion. The COCs with mean diameter of 216.93±6.28 µm were cultured in this group. There was a minimal increase in COCs diameter at 8 (223.06±5.56µm), 16 (224.88±7.27µm) and 24 (226.36±6.95µm) hours of culture period. At the end of culture period of 24 hours there was only 9.43±3.89µm non significant ($P>0.05$) increase in mean diameter of COCs.

Effect of oestradiol and rhFSH on cumulus expansion

The stimulatory effect of rhFSH on cumulus expansion was further enhanced when E_2 was added in culture medium along with rhFSH (0.05IU/ml). The mean diameter of COCs at 0 hours was 191.41±6.23 µm which increased to 272.608±6.5 µmat 8 hours, 347.71±5.19 µm at 16 hours and 395.0±4.29 µm at 24 hours. The combined effect of oestradiol and rhFSH resulted in highly significant ($P<0.0001$) cumulus expansion of 203.60±6.36 µm at the end of maturation period compared to the cumulus expansion in control group.

Effect of oestradiol and hCG on cumulus expansion

Culturing of COCs in the presence of E_2 + hCG (2IU/ml) was observed to have no effect on cumulus expansion. The diameter of COCs observed at different time period in this treatment group was 222.0±6.55µm (0 hours), 227.0±4.88µm (8 hours), 242.25±6.10µm (16 hours), 255.25±5.32 µm (24 hours). The net increase in the mean diameter was 33.25±7.14µm, which was not significant ($P > 0.05$) compared to control group.

Effect of oestradiol and insulin on cumulus expansion

The addition of insulin with oestradiol in culture medium stimulated the cumulus expansion.

The initial diameter of COCs in this group was 219.84±4.21 µm at 0 hours, 234.68±3.81 µm at 8 hours, 249.84±6.40 µm at 16 hours and 269.68±4.23 µm 24 hours. The total increase in the diameter of COCs was 49.84±4.63 µm. Although there was an increase in mean diameter of COCs but this was not significant ($P>0.05$) compared to the control group.

Table: Effect of different combinations of oestradiol (E2), Recombinant Human Follicle Stimulating Hormone (rhFSH), Human Chorionic Gonadotrophin (hCG) and Insulin on the expansion of cultured Nili Ravi buffalo COCs for a period of 8,16 and 24 hours.

Treatment group	Diameter (µm) of COCs observed at different incubation time intervals				
	0 Hours	8 Hours	16 Hours	24 Hours	Expansion of COCs (µm)
Control (N=10)	202.5±8.32	205.75±8.66	208.25±5.89	216.75±9.10	24.25± 7.32
Oestradiol (N=15)	233.36±6.68	257.66±9.12	267.166±9.95	276.16±10.2	42.79±8.82
rhFSH (N=12)	202.08±3.28	223.33±3.82	276.66±5.80	352.08±11.5	150±11.20
hCG (N=10)	244.25±8.53	245.75±5.12	250.5±4.07	271.75±7.3	27.50±8.31
Insulin (N=22)	216.93±6.28	223.06±5.56	224.88±7.27	226.36±6.95	9.43±3.89
E_2+rhFSH (N=23)	191.41±6.23	272.608±6.5	347.71±5.19	395.0±4.29	203.60±6.36
E_2+hCG (N=10)	222.0±6.55	227.0±4.88	242.25±6.10	255.25±5.32	33.25±7.14
E_2+Insulin (N=16)	219.84±4.21	234.68±3.81	249.84±6.40	269.68±4.23	49.84±4.63
rhFSH+hCG (N=11)	224.31±4.51	226.36±4.46	244.31±4.22	256.36±5.57	32.05±6.54
rhFSH+Insulin (N=22)	205.34±2.23	211.70±2.61	218.52±3.70	249.77±8.13	44.43±8.30
hCG+Insulin (N=18)	224.44±5.71	228.33±6.86	236.25±8.95	252.08±8.05	27.64±8.88
E_2+rhFSH+hCG (N=22)	231.25±4.11	235.0±4.42	276.36±4.37	311.59±6.22	80.34±8.42
E_2+rhFSH+hCG+Insulin (N=23)	211.19±3.80	223.04±5.75	233.04±4.70	242.39±4.17	31.20±4.09

Values in table are Mean ± SEM N= Number of COCs

Effect of rhFSH and hCG on cumulus expansion

The stimulatory effect of rhFSH on cumulus expansion was suppressed by the addition of hCG in culture medium. COCs with mean diameter of 224.31±4.51 µm were incubated and the diameter of COCs observed at different time intervals was 226.36±4.46 µm at 8 hours, 244.31±4.22µm at 16 hours, 256.36±5.57 µm at 24 hours. There was not significant ($P > 0.05$) increase of 32.05±6.54 µm in mean diameter of COCs after 24 hours of incubation period.

Effect of rhFSH and insulin on cumulus expansion

Addition of rhFSH and insulin in a medium also showed the same results as showed by rhFSH +hCG but this combination was comparatively better. The COCs with 205.34±2.23 µm become 211.70±2.61 µm at 8 hours, 218.52±3.70µm at 16 hours and 249.77±8.13 µm 24 hours of maturation period. The not significant ($P>0.05$) increase in the diameter of COCs was obtained as 44.43±8.30 µm.

Effect of hCG and insulin on cumulus expansion

Combined effect of insulin with hCG in a culture medium did not stimulated the cumulus expansion. The diameter of COCs observed in this group was 224.44±5.71 µm at 0 hours, 228.33±6.86 µm at 8 hours, 236.25±8.95 µm at 16 hours and 252.08±8.05 µm at 24 hours. The net increase in mean diameter was 27.64±8.88 µm after 24 hours and this was a non significant ($P>0.05$) compared to the control group.

Effect of oestradiol, rhFSH and hCG on cumulus expansion

The maximum expansion obtained in combination of oestradiol and rhFSH was reduces when hCG was added in a similar combination. The diameter observed in this group was 231.25±4.11 µm at 0 hours, 235.0±4.42 µm at 8 hours, 276.36±4.37 µm at 16 hours and 311.59±6.22 µm at 24 hours. At the end of culture period 80.34±8.42 µm cumulus growth was achieved in this treatment group. The cumulus growth in this treatment group was not significant ($P > 0.05$) compared to the growth of the cumulus in control group.

Effect of oestradiol, rhFSH, hCG and insulin on cumulus expansion

The combined effect of E_2, rhFSH, hCG and insulin in TCM-199 was also examined. The COCs with initial mean diameter of 211.19±3.80 µm were selected. The diameter of COCs was 223.04±5.7 at 8 hours, 233.04±4.70 at 16 hours and 242.39±4.17 µm at 24 hours. The total increase in the mean diameter of COCs was 31.20±4.09 µm. This increase in the mean diameter of the COCs was not significant ($P>0.05$) compared to the increase in the diameter of COCs in control group.

DISCUSSION

In the present study various hormones were used to find out which combination of hormones improves the cumulus expansion in Nili Ravi buffalo COCs, since optimal expansion of the cumulus mass is essential for cytoplasmic maturation.

The present study revealed that although the cumulus expansion was stimulated by E_2 supplementation. Similar increase of cumulus expansion has also been reported in Nili Ravi buffalo COCs when matured in oocyte maturation medium contained 0.95% TCM -199; 0.002% estradiol; 0.001% hCG; 0.04% BSA; 100µl fetal calf

serum; 2×10^{-4} mol sodium pyruvate and 0.05% gentamicin (Aziz *et al.,* 2012).

The supplementation of rhFSH in this study stimulated the cumulus expansion at 8, 16 and 24 hours of culture. This study is similar to another study in which it was reported that cumulus expansion of bovine oocytes was maximal (complete expansion) in the presence of rhFSH with BSA and intermediate (partial expansion) in the presence of polyvinyl pyrrolidone 40 (PVP-40) + rhFSH (Ali and Sirard, 2002). Similarly in another study the cumulus expansion reported in COCs cultured at doses of 1, 100 and 1000 ng/ ml rhFSH or porcine FSH compared to negative control , and after 21 to 22 hours of maturation and a significant effect was observed due to treatment ($P<0.001$; Calder *et al.,* 2003). All doses of rhFSH tested caused significantly greater expansion of bovine COCs compare with 0ng/ml. It was studied by Nagyova (2012) that FSH or 8-bromo Cam Pinduce the cumulus expansion. The glutamine and glucose are present in TCM199 commonly used for *in vitro* maturation of bovine oocytes, similarly in this study, TCM199 with both glutamine and glucose has been used. Hence the adequate concentration of glucose and glutamine induced expansion with rhFSH. In another study it was reported that the only 1 ng/ml rhFSH in serum free media induces the cumulus expansion in bovine COCs (Calder *et al.,* 2003).

In present study the addition of hCG in culture media failed to induce the cumulus expansion till 16 hours of culture however a small stimulatory effect on cumulus expansion was observed at 24 hours. In a similar study young *et al* (2001) reported that the low doses of hCG failed to affect the oocytes maturation and cumulus expansion of cumulus enclosed oocytes.

The insulin supplementation did not show significant changes in cumulus expansion in present study. The results of our study are in agreement with another study in which Kun *et al.,* 2009 reported that the addition of the insulin to *in vitro* maturation medium of canine oocytes had no effect on oocytes maturation and cumulus expansion. Similarly, in another study insulin (10mg/ml) had no effect on bovine oocytes maturation, fertilization and cleavage rates (Matsui *et al.,* 1995).

Supplementation of E_2 and rhFSH with 0.4% bovine serum albumen in TCM199 caused maximum increase in the cumulus expansion of *in vitro* matured follicle after 8, 16 and 24 hours of culture. In present study it was observed that the stimulatory effect of rhFSH on cumulus expansion was further improves by the addition of E_2. Similarly in another study it was reported that the there was significant increase in cumulus expansion in the presence of oestradiol, regardless of the concentration, during pre-incubation compared with the oestradiol-free medium (Nakakoji and Funahashi, 2012). The present study revealed that when medium was supplemented with rhFSH and hCG there was no cumulus expansion at 8 hours of culture period. The stimulatory effect of rhFSH on cumulus expansion was decreased by the addition of hCG. However at 16 and 24 hours of culture period, cumulus expansion was observed. Ji Wu *et al.* (2000) reported that the effect of optimal concentration of FSH, LH and high serum on growth of mice follicles from 120-140 μm sizes. Present study is similar to another finding in which it was reported that FSH together with insulin increased the follicular diameter in bovine preantral follicle (Gutierrez *et al.,* 2000).

In this study the addition of hCG also depressed the combined effect of E_2, rhFSH in maturation medium on cumulus expansion at 8 hours of incubation period, however, this combination induced the cumulus expansion after 16 hours of culture period. The similar effect on cumulus expansion was observed in E_2, rhFSH, hCG and insulin combination. Bovine insulin (1-1000 ng/ml) had no effect on *in vitro* maturation when bovine oocytes cultured in TCM199containing fetal calf serum, gonadotrophins and E_2 (Stubbings *et al.,* 1990).This may reflect the species-species difference in the cumulus expansion mechanism.

CONCLUSION

It was concluded that presence of hormones in TCM199 enhances the cumulus expansion of COCs in Nili Ravi buffalo during culture *in vitro*. Supplementation of medium with single hormone shows maximum expansion in COCs with rhFSH after 24 hours. Addition of double hormones combination also has positive effect on cumulus expansion of COCs. But the significant increase in mean diameter of COCs was observed with E_2+rhFSH even at 8 hours of incubation.

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