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Research Article

IN VITRO MATURATION OF BUFFALO OOCYTES IN SERUM FREE MEDIA SUPPLEMENTED WITH GROWTH FACTORS

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ABSTRACT

The present study was performed to evaluate the effect of growth factors (IGF-1 and EGF) supplementation into serum free media on *in vitro* maturation of buffalo oocytes derived from abattoir ovaries. A total of 792 cumulus oocytes complexes (grade A + B) derived from 580 buffalo ovaries were *in vitro* matured in five different groups *viz.* Group 1: Control (TCM-199), Group 2: TCM-199 + IGF-1@50ng/ml, Group 3: TCM-199 + EGF@10ng/ml, Group 4: TCM-199 +IGF-1@50ng/ml + EGF@10 ng/ml and Group 5: TCM-199 + Fetal Bovine Serum (FBS) @10 percent. The hormones (FSH 0.5 IU/ml, LH 5 IU/ml, Estradiol 1µg/ml); Cysteamine @50µM and Gentamicin @ 50µg/ml were also supplemented in all groups. Oocytes were cultured in CO_2 incubator (22-24 h) at 38.5 ºC, using 5% CO_2 and high relative humidity. Maturation of oocytes was assessed by cumulus cell expansion and Orcein nuclear staining (0.1%). The study showed, supplementation of IGF-1 (group 2) and EGF (group 3) alone did not significantly improve cumulus cell expansion and nuclear maturation as compared to control group (72.6% and 75.8% vs. 69.0 and 57.8 and 56.4 vs. 43.4 percent, respectively). The percentage of oocytes with fully expanded cumulus cells and reaching metaphase-II stage were significantly higher ($P<0.5$) in groups 4 and 5 than any other group. The results concluded that the supplementation of IGF-1 + EGF could be used to substitute serum for serum free in vitro maturation of immature buffalo oocytes.

Keywords: Buffalo, EGF, IGF-1, *In vitro* maturation, Oocytes

In vitro production of embryos (IVP) is more efficient in increasing the number of transferable embryos per unit time compared to conventional superovulation based embryo transfer technique. The yield of transferable embryos and birth of live calves following IVP are still very low in buffaloes (Prasad *et al.*, 2013). The first and most critical step towards successful IVP is development of a suitable oocyte maturation system. Most of the *in vitro* maturation (IVM) protocols involve supplementation of serum and different hormones (FSH, LH and Estradiol) to obtain high maturation and embryo development rates in cattle and buffaloes. However, presence of serum in media is considered as a source of embryonic abnormalities (Barnes, 2000). Therefore, the establishment of serum free defined media is essential to evaluate the role of various supplements during IVP and to overcome the undesired effects of serum.

Supplementation of maturation media with growth factors viz. insulin like growth factor-1 (IGF-1) and epidermal growth factor (EGF) improved oocyte maturation and developmental competence in the presence of serum (Sadeesh *et al.*, 2014). However, the efficacy of IGF-1 and EGF during IVM of buffalo oocytes in the absence of serum is scarce (Kumar and Purohit, 2004). Therefore, the present study was planned to evaluate the effect of supplementation of IGF-1 and EGF in serum free media on *in vitro* maturation rates of immature buffalo oocytes collected from abattoir ovaries.

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MATERIALS AND METHODS

All media and chemicals used were obtained from Sigma Aldrich Chemical Co. Ltd, St. Louis, MO, USA.

(i) Collection of ovaries and oocytes: Buffalo ovaries (n=580) were collected in normal saline containing Gentamicin 50 µg/ml, maintained at 30-32 ºC and brought to laboratory within 4 hours of slaughter. Visible follicles of >2 to < 8 mm diameter were aspirated by 18 gauge needle attached to 10 ml syringe. Based on number of cumulus cell layers and homogeneity of cytoplasm, the recovered oocytes were graded as described by Singhal *et al.* (2009); Grade A - COCs with 4 or more layers of compact cumulus cells investment, evenly granular homogenous ooplasm, light and transparent in appearance; Grade B - COCs with 2-3 layers of compact cumulus cells investment, evenly granular homogenous ooplasm; Grade C - COCs with 1 layer of cumulus cells investment; and Grade D - Partially or completely denuded COCs or COCs with expanded cumulus investment, highly scattered cumulus cells, irregular dark ooplasm.

(ii) *In vitro* maturation (IVM) of oocytes: A total of 792 culturable oocytes (grade A + B) were matured in 5 different treatment groups *viz.* Group 1 Control (TCM-199), Group 2 TCM-199 + IGF-1@ 50 ng/ml, Group 3 TCM-199 + EGF@ 10 ng/ml, Group 4 TCM-199 + IGF-1@ 50ng/ml + EGF@10 ng/ml and Group 5 TCM-199 + Fetal Bovine Serum 10%. Hormones (FSH 0.5 IU/ml, LH 5 IU/ml, Estradiol 1µg/ml); Cysteamine @ 50µM and Gentamicin @ 50µg/ml were also added to all the groups.

Oocytes were cultured in already incubated droplets (20 oocytes per 100µl droplets) covered under mineral oil in 35x10 mm petridishes (Falcon) for 22 to 24 h in CO_2 incubator (Model-3131, Thermo Fisher Scientific, USA) maintained at 38.5 ºC, with 5 per cent CO_2 and high relative humidity.

(iii) Orcein staining of matured oocytes: Matured oocytes were stained and evaluated as described by Hunter and Polge (1966). Briefly, oocytes were denuded using 0.1% (w/v) hyaluronidase in calcium and magnesium free Dulbecco's Phosphate Buffer Saline (DPBS). Five to ten denuded oocytes were mounted on glass slide under coverslip (supported with paraffin-vaseline corners) and fixed in ethanol : acetic acid (3:1, v/v) for 24 hours. Later these oocytes were stained in 1% orcein (w/v) in 45% acetic acid (v/v) for 20 min and differentiated by gently running differentiation solution (acetic acid : distilled water : glycerol :: 1 : 3 : 1) between the slide and coverslip.

Parameters studied

The *in vitro* maturation was assessed by the degree of expansion of cumulus cells and by orcein staining after 22-24 hours of incubation.

(i) Cumulus cells expansion

The *in vitro* maturation of oocytes was assessed based on the degree of cumulus cell expansion and classified as per Hunter and Moor (1987). Accordingly, Grade-1: Full cumulus cell expansion (enlargement of the cumulus cell mass at least 3x diameter away from the zona pellucida); Grade-2: Moderate cumulus cell expansion (expansion in order of 2x diameter away from the zona pellucida); Grade-3: Slight expansion of cumulus cells (no evident change).

(ii) Nuclear maturation

The orcein stained oocytes were evaluated under a phase contrast microscope at 200X and were classified into germinal vesicle (GV), Intermediate and Metaphase II (M II) stage based on the nuclear maturation.

Statistical analysis

The data of *in vitro* matured oocytes were expressed in percent and analyzed using chi-square test by SPSS-16. P value at 5% was considered as significant.

RESULTS AND DISCUSSION

The results of present study showed that the groups supplemented with IGF-1, EGF, IGF-1 + EGF or serum had higher percentage of full cumulus cell expansion compared to the control (Table 1). In groups 2 and 3 supplemented with IGF-1 and EGF, respectively, cumulus cell expansion was non-significantly higher than control

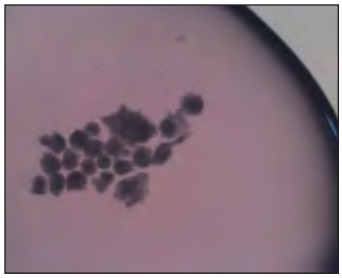

Fig. 1. Immature oocytes retrieved from abattoir buffalo ovaries

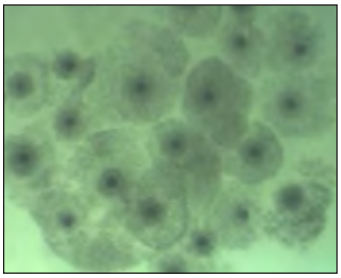

Fig. 2. Cumulus cell expansion following 22 hrs Cocs culture

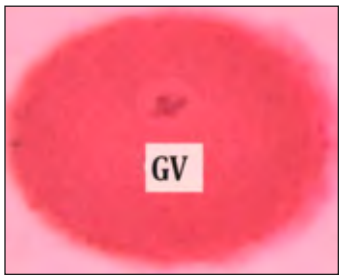

Fig. 3. Germinal Veside (GV) Stage

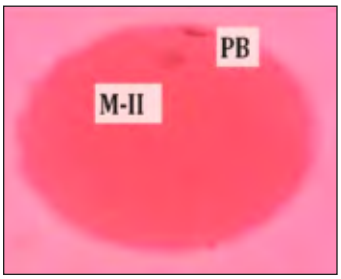

Fig. 4. Metaphase II (M II) Stage

but lower than groups 4 and 5. Highest percentage of oocytes having fully expanded cumulus cells was observed in group 5 followed by the group 4; however, the difference was non-significant indicating that supplementation of TCM-199 with IGF-1 and EGF was effective in promoting oocyte maturation as indicated by cumulus cell expansion.

Results of the present study were in agreement with Chohan and Hunter (2003) and Kumar and Purohit (2004) who observed no significant difference in *in vitro* maturation of oocytes cultured in serum free or serum supplemented media. Cevik *et al.* (2011) reported higher cumulus expansion rate of 85.5% and 87.4% in TCM-199 media supplemented with hormones and EGF with and without FBS, respectively. Sadeesh *et al.* (2014) achieved higher cumulus cell expansion rate of 94.4% using TCM-199 supplemented with EGF (20 ng/mL) + β - ME (100 µ M) in the presence of 10% FBS and hormones. Compared to the present study, Nagar and Purohit (2005) found that cumulus expansion of oocytes increased significantly with EGF supplementation in a dose-dependent manner up to 50 ng/ml and proportion of oocytes reaching M-II upto 20 ng/ml.

The orcein staining of oocytes showed that higher proportion of oocytes reached M-II stage in all the supplemented groups compared to the control (Table 2). The proportion of oocytes reaching M-II stage did not differ between group-2 and group-3 (57.8 % vs 56.4 %; $P<0.05$) but it was lower than groups 4 and 5 (57.8 % & 56.5 % vs 69.2 % & 72.5 %; $P<0.05$, respectively). In group-4, the additive effect of combining IGF-1 and EGF was visible and higher proportion of oocytes reached

Table 1

Effect of supplementation of IGF-1, EGF and serum to *in vitro* maturation media on cumulus cell expansion

Parameter	No. of ovaries	No. of oocytes cultured	No Expansion	Partial Expansion	Full Expansion
			Number (%)	Number (%)	Number (%)
Group-1 (Control: TCM)	109	142	19 (13.4)[a]	25 (17.6)[a]	98 (69.0)[a]
Group-2 (TCM+IGF-1)	116	157	21 (13.4)[a]	22 (14.0)[a]	114 (72.6)[ab]
Group-3 (TCM+EGF)	112	153	19 (12.4)[a]	18 (11.8)[ab]	116 (75.8)[ab]
Group-4 (TCM+IGF-1+EGF)	115	162	10 (6.2)[b]	15 (9.3)[b]	137 (84.6)[c]
Group-5 (TCM+FBS)	128	178	11 (6.2)[b]	14 (7.9)[b]	154 (86.5)[c]

Values marked with different superscript in a same column differ significantly at 5% level.

Table 2

Effect of IGF-1, EGF and serum supplementation on nuclear maturation assessed by orcein staining

Parameter	No. of oocyte stained	GV stage	Intermediate stage	Metaphase-II
		Number (%)	Number (%)	Number (%)
Group-1 (Control; TCM -199)	75	15 (20.0)[a]	28 (37.3)[a]	32 (42.7)[a]
Group-2 (TCM+IGF-1)	90	9 (10.0)[a]	29 (32.2)[a]	52 (57.8)[ab]
Group-3 (TCM+EGF)	94	14 (14.9)[a]	27 (28.7)[ab]	53 (56.4)[ab]
Group-4 (TCM+IGF-1+EGF)	104	11 (10.6)[a]	21 (20.1)[b]	72 (69.2)[bc]
Group-5 (TCM+FBS)	120	14 (11.7)[a]	19 (16.6)[c]	87 (72.5)[bc]

metaphase II compared to the groups 1, 2 and 3.

Gupta *et al.* (2002) and Singh (2006) compared various concentrations of EGF (10, 20, 30 ng/ml) in TCM-199 and reported higher maturation rate with 20 ng/ml (80%) as compared to 10 ng/ml (58%) or 30 ng/ml EGF (76%). However, Lonergan *et al.* (1996) and Bastan *et al.* (2010) did not find dose dependent effect of EGF on oocyte maturation rates.

Significantly, higher maturation rates (78.04 and 83.52%) in Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-10 supplemented with a combination of EGF + IGF-1 have been reported by Kumar and Purohit (2004) compared to 69.2% recorded in group-4 (supplemented with IGF-1 +EGF) of the present study. Similarly, Purohit *et al.* (2005) also reported higher nuclear maturation rate of 64.7%, 63.2% and 81% compared to 57.8%, 56.4% and 69.2% observed in present study with IGF-1, EGF and EGF+IGF-1, respectively.

The addition of IGF-1 to maturation media also showed stimulatory effect on nuclear maturation. Pawshe *et al.* (1998) observed that the IGF-1@100 ng/ml concentration is a major follicular factor responsible for stimulating oocyte maturation in buffaloes. Singhal *et al.* (2009) reported that IGF-I and cysteamine together improved the IVP rate of oocytes collected *in vivo* from buffalo.

The experiment showed that the proportion of oocytes with fully expanded cumulus cells or reaching M-II stage was highest in Groups 4 and 5 compared to other treatment groups in this study. Cumulus cells expansion and nuclear maturation rate did not differ between Group-4 supplemented with IGF-1+EGF (without serum) and Group-5 supplemented with serum hormones. Therefore, it indicated that the serum in maturation medium could be substituted with IGF-1+EGF without affecting the oocytes nuclear maturation.

CONCLUSION

From the results, it could be concluded that that the TCM 199 supplemented with IGF-1 + EGF may be used as serum free media for *in vitro* maturation of buffalo oocytes collected from abattoir ovaries. However, further studies are required to assess the subsequent developmental competence of buffalo oocytes matured with supplementation of IGF-1 + EGF in serum free *in vitro* maturation media.

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