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RESEARCH ARTICLE

Effect of Somatic Cell Types and Culture Medium on *in vitro* Maturation, Fertilization and Early Development Capability of Buffalo Oocytes

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ABSTRACT

This study was designed to evaluate the efficacy of different somatic cell types and media in supporting *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and early embryonic development competence of buffalo follicular oocytes. Cumulus oocyte complexes were collected for maturation from follicles (>6mm) of buffalo ovaries collected at the local abattoir. Oocytes were co-cultured in tissue culture medium (TCM-199) with either granulosa cells, cumulus cells, or buffalo oviductal epithelial cells (BOEC) @ $3x10^6$ cells/ml or in TCM-199 without helper cells (control) at 39°C and 5%CO_2 in humidified air. Fresh semen was prepared in modified Ca^{++} free Tyrode medium. Fertilization was carried out in four types of media: i) Tyrode lactate albumin pyruvate (TALP), ii) TALP+BOEC, iii) modified Ca^{++} free Tyrode and iv) modified Ca^{++} free Tyrode+BOEC. Fertilized oocytes were cultured for early embryonic development in TCM-199 with and without BOEC. Higher maturation rates were observed in the granulosa (84.24%) and cumulus cells (83.44%) than BOEC co culture system (73.37%). Highest fertilization rate was obtained in modified Ca^{++} free Tyrode with BOEC co culture (70.42%), followed by modified Ca^{++} free Tyrode alone (63.77%), TALP with BOEC (36.92%) and TALP alone (10.94%). Development of early embryos (8-cell stage) improved in TCM-199 with BOEC co culture than TCM-199 alone. From the results of this study, it can be concluded that addition of somatic cells (granulosa cells, cumulus cells) results in higher maturation rates of buffalo follicular oocytes than BOEC co culture system, while fertilization rate improved in modified Ca^{++} free Tyrode with and without BOEC. Addition of BOEC to TCM-199 improved the developmental capacity of early embryo.

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INTRODUCTION

Buffalo is the main dairy animal in Pakistan and accounts for approximately 75% of all milk produced in the country. Inherent reproductive problems, such as delayed puberty (Nandi *et al*., 2002), delayed first calving (Lundstrom *et al*., 1982), late postpartum conception (Arora and Jain, 1988) and longer calving intervals (Singh and Roy, 1996) lead to low reproduction and production performance. Genetic improvement in this important animal resource is focused on its role as a major provider of milk and meat in this part of the world. Limitation to genetic improvement imposed by inherent biological parameters can be overcome by the use of recently developed reproductive biotechnologies. *In vitro* production of buffalo embryos adopting the procedures developed for cattle has received increasing interest in recent years. The efficiency of *in vitro* embryo production **(**IVEP) in buffalo is much lower than in cattle in terms of IVM, IVF and yield of transferable quality embryos (Madan *et al*., 1994; Palta and Chauhan, 1998). Nandi *et al*. (2002) reported that the *in vitro* embryo production system in buffalo is suboptimal and requires substantial improvement along with several other problems that need to be resolved before *in vitro* embryo production technology can be used regularly in buffalo breeding. In mammals, somatic cell-oocytes communication provides beneficial effects by providing nutritional molecules that are necessary for growth and development and by transmitting signals that regulate oocytes maturation

(Eppig *et al*., 1983). To optimize IVEP of buffalo embryos by improving the culture conditions the present project was designed to assess the effect of addition of somatic cells (i.e. follicular granulosa, cumulus and oviductal epithelial cells) on IVM, IVF and development of fertilized buffalo follicular oocytes.

MATERIALS AND METHODS

Collection of oocytes

Ovaries from sexually mature buffaloes were collected within 30min after slaughter from the local abattoir and transported to the laboratory in PBS (pH 7.35) supplemented with 100IU/ml penicillin G and 100µg/ml streptomycin maintained at a temperature of 25-30°C (Totey *et al*., 1992). Extraneous tissue was removed and ovaries were washed with 70% ethanol, followed by three rinses in PBS. Oocytes were collected by scoring method (Suss and Madison, 1983). Oocytes possessing a full cumulus mass, unfragmented cytoplasm and intact zona were selected for further processing. About 1.48±0.53 on an average good quality oocytes per buffalo ovary were collected and it took about two hours from slaughtering to putting them into IVM medium.

Collection of somatic cells

In the present study, three types of somatic cells viz. follicular granulosa cells, cumulus cells and BOEC were used. Follicular granulosa cells were collected and prepared according to the method described by Lu *et al.* (1987). For collection and preparation of cumulus cells from oocytes the method described by Pawshe and Totey (1993) was followed and method described by Eyestone and First (1989) was used for collection and preparation of oviductal epithelial cells. Collected somatic cells ability was determined via trypan blue staining. The samples containing high percentages of dead cells were discarded (Freshney, 1991).

In vitro maturation

For maturation studies, the tissue culture medium TCM-199 (Sigma, St. Louise USA) supplemented with 20% estrus buffalo serum (EBS) and gentamycin 10 µg/ml (Sigma) at pH 7.4 was used. Selected oocytes were washed twice in maturation medium and 10-15 oocytes were incubated in 200 µl of medium. A total of 4 drops of the 200 µl maturation medium, were prepared in 60x35mm sterile Petri dishes. Experimental somatic cells were added @ $3x10^6$/ml to each drop while one drop served as control. The drops in Petri dishes were covered with sterile mineral oil (Sigma USA) and were placed in a CO_2 incubator at 39°C and 5% CO_2 for 24h. At the end of maturation period under oocytes were checked stereomicroscope. Oocytes having expanded cumulus cells with extrusion of the polar body were considered matured. Following IVM, some oocytes from somatic cell supplementation and control groups were fixed and stained for assessment of nuclear maturation (Jainudeen *et al*., 1993).

In vitro fertilization and early embryo development

For IVF studies, modified Ca^{++} free Tyrode's and TALP media (pH 7.8) were used. Oocytes matured in granulosa and cumulus co-culture systems were used for *in vitro* fertilization. Fresh, good quality semen from a buffalo bull was used for insemination of matured oocytes. For capacitation, final sperm suspension was diluted with 1 ml sperm washing medium containing 21.87 IU/ml heparin (Sigma) and incubated for 15 min. The sperm concentration was adjusted to $1x10^6$/ml (Parrish *et al*., 1985) for insemination of oocytes. Droplets (50µl) of four types of fertilization media were prepared i) TALP, ii) TALP+BOEC, iii) modified Ca^{++} free Tyrode medium and iv) modified Ca^{++} free Tyrode+BOEC. BOECs were added to these media at a concentration of $3x10^6$/ml.

Oocytes (5-10/drop) and sperm cells were co-incubated at 39ºC with 5% CO_2 in humidified air for 24h. After co-incubation, the inseminated oocytes were transferred to 200 µl of TCM-199 supplemented with BOEC. A control culture lacking BOEC was established. Only oocytes fertilized in Ca^{++} free tyrode medium were cultured in TCM-199 at 39ºC in 5%CO_2 in humidified air for early development. The culture medium was changed every two days and embryo development up was recorded.

Statistical analysis

The data thus obtained was subjected to statistical analysis through statistical software package SAS using Logistic Regression Analysis. The percentages of oocytes reaching the designated response variable in each replicate were determined. The data on IVM and IVF was subjected to FREQ Procedure. GENMOD Procedure was used for assessing Differences of Least Squares Means and Contrast. The percentages of fertilized oocytes reaching eight cell stages were analyzed by Test and Confidence Interval for two proportions.

RESULTS

The effect of supplementation of culture medium with somatic cells on maturation rate is shown in Table 1. Results vary significantly between treatments (P=0.0438). Addition of granulosa and cumulus cells improved the maturation rate (P=0.0162 and P=0.0290, respectively) compared to BOEC group. Effect of fertilization media and addition of somatic cells on fertilization rate is shown in Table 2. The fertilization rate was higher in modified Ca^{++} free Tyrode+BOEC medium as compared to other treatment groups. Addition of BOEC significantly improved the fertilization rate in TALP (P=0.001) the fertilization media. The developmental capacity of cleaved oocytes to reach 4 & 8-cell stages in the presence and absence of BOEC is shown in Table 3. Addition of BOEC improved developmental capacity compared to the medium alone.

DISCUSSION

In vitro embryo production in buffaloes is not yet an established technology. The initial success of embryo transfer in Riverine buffalo in the USA (Drost *et al.*, 1983) was followed by the birth of embryo transfer calves in Bulgaria (Vlanov *et al*., 1985) and India (Singh *et al.*, 1989). Initial successes involved the transfer of embryos derived *in vivo*. However, the efficiency of *in vivo* embryo

production in water buffalo has not improved much over the years. To develop culture media for buffalo IVEP, attempts have been made by partially or completely replace the basics medium (TCM-199) with cystic or follicular fluid (Gupta *et al.,* 2001) or with fetal bovine serum (Chauhan *et al.,* 1998). Investigation in cattle resulted in the development of oviductal co-culture system for buffalo which improved the cleavage rate (Pawshe and Totey 1993; Madan *et al.,* 1994). The present study was an attempt to improve the maturation rate, IVF, and subsequent embryonic development in the presence of somatic cells added to the medium.

Table 1: Effect of somatic cells supplementation of TCM-199 on *in vitro* maturation rate of buffalo follicular oocytes

Somatic cells ($3x10^6$/ml)	No. of oocytes		
	Total	Matured	Not Matured
Control	191	148 (77.49)	43 (22.51)
Follicular Granulosa	165	139 (84.24)	26 (15.76)
Cumulus Oophorus	157	131 (83.44)	26 (16.56)
BOEC	169	124 (73.37)	45 (26.63)
Total	682	542	140

χ^2=8.13; P=0.0434; Figures in parenthesis indicate percentage.

Table 2: Effect of fertilization media on fertilization rate of buffalo follicular oocytes

Media	Total	Fertilized	Unfertilized
Fertilization Media No. 1 (χ^2=11.92; P=0.000)			
TALP	64	7 (10.94)	57 (89.06)
TALP+BOEC	65	24 (36.92)	41 (63.08)
Total	129	31	98
Fertilization Media No. 2 (χ^2=0.70; P=0.400)			
Modified Ca^{2+}free Tyrode's	69	44 (63.77)	25 (36.23)
Modified Ca^{2+}free Tyrode's +BOEC	71	50 (70.42)	21 (29.58)
Total	190	94	46

Figures in parenthesis indicate percentage.

Table 3: Effect of culture media on development capacity of in vitro fertilized/cleaved buffalo follicular oocytes

Media	No. of Developmental Stages		
	2 Cells	4 Cells	8 Cells
TCM-199	48	15 (31.25)	10 (20.83)
TCM-199+BOEC	50	20 (40.0)	14 (28.0)

Figures in parenthesis indicate percentage.

In this study, a higher maturation rate of buffalo oocytes was achieved in the presence of follicular granulosa and cumulus cells in the maturation medium. The results are in agreement with the findings of Suh *et al.* (1993) and Galli and Lazzari (1996). Critser *et al.* (1986) have reported that granulosa cells interact with cumulus oocyte complex and are involved in improving the developmental capacity of bovine and ovine oocytes respectively. Durnford *et al.* (1994) reported that the beneficial effect of somatic cells is mediated by their contribution of some specific component to the culture medium or the removal of inhibitory substances. In the present study the maturation rates were similar with the addition of follicular granulosa to cumulus cells. Mochizuki *et al.* (1991) used the follicular granulosa and cumulus cells in cattle and reported a slightly higher maturation rate with follicular granulose compared to cumulus. King *et al.* (1990) and Fassi-Fihri *et al.* (1991) also reported the beneficial effect of the presence of follicular granulosa and cumulus cells in the culture medium.

In the present study, addition of BOEC did not improve the maturation rate. These findings are also in agreement with Gliedt *et al.* (1996). This could be due to the fact that oviductal cells provide a favorable environment for fertilization of oocytes. The somatic cells density ($3x10^6$/ml) used in the present study have also been reported earlier by Leibfried-Rutledges *et al.* (1989) in cow and by Staigmiller and Moor (1984) in sheep. These studies further mentioned that the developmental competence of ooytes was reduced with the reduction in the number of somatic cells.

In the present study a higher fertilization rate was achieved by modified Ca^{++} free Tyrode's medium as compared to TALP. It was further noticed that the supplementation of fertilization medium with BOEC significantly improved the fertilization rate in TALP medium (P=0.001). The higher fertilization rate in Ca^{++} free Tyrode medium in the present study correlated with the findings of Behnke (1987) and Ijaz and Hunter (1989) who reported that capacitation of sperm at pH 7.6 in Ca^{++} free Tyrode's medium resulted in a higher penetration rate zona. They also reported that this medium stimulates the sperm to undergo changes that preceded the acrosome reaction and pH of the medium precluded the necessity for calcium. Significant enhancement of the fertilization rate in the presence of BOEC was also supported by the findings of Trounson *et al.* (1977), who further reported that sperm attachment to the oviductal cell confers preservation of sperm viability and that cellular secretion is able to induce the changes related to sperm acquisition of ability to interact and penetrate the oocyte. No earlier work in Ca^{++} free medium in the presence of BOEC is known to the author.

Enhanced developmental competence of embryos in the presence of BOEC in the present study is in agreement with the findings of Kim *et al.* (1990), who reported beneficial effect of BOEC supplement in the culture media in cattle. Gandolfi and Moor (1987) reported that. Madan *et al.* (1994) also reported a higher development rate of embryos in the presence of BOEC.

In conclusion, the addition of follicular granulosa cells and cumulus cells result in a higher maturation rate of buffalo follicular oocytes than addition of oviductal epithelial cells. A significantly higher fertilization rate was obtained for modified Ca^{++} free Tyrode's+BOEC media than in TALP and TALP+BOEC medium. Addition of BOEC to TCM-199 improved the developmental capacity of early embryo compared to TCM-199 alone.

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