EFFECT OF TCM-199 AND SYNTHETIC OVIDUCTAL FLUID (SOF) MEDIUM AND CYSTEAMINE SUPPLEMENTATION TO *IN VITRO* MATURATION MEDIA ON MATURATION, CLEAVAGE RATE AND SUBSEQUENT EMBRYONIC DEVELOPMENT OF BUFFALO OOCYTES

Ch. Srinivasa Prasad*, A. Palanisamy, V.S. Gomathy, S. Satheshkumar, A. Thangavel and G. Dhinakar Raj

ABSTRACT

The present study has been undertaken to study the effect of TCM-199 and synthetic oviductal fluid (SOF) medium and the influence of cysteamine (100 µM) supplementation to IVM medium on maturation, cleavage and subsequent embryonic development of buffalo oocytes. In the present study it was observed that buffalo oocytes showed no significant variation in the percent of cumulus cell expansion when matured in TCM-199 and SOF media. The mean percent of cleavage rate and subsequent development to morula in buffalo oocytes matured in TCM-199 and SOF media were 28.06 ± 2.19, 10.20 ± 0.91 and 28.99 ± 0.66, 10.65 ± 0.40, respectively. There was no significant difference with regard to cleavage rate and subsequent development to morula when buffalo oocytes were matured in TCM-199 and SOF media.

The percentage of oocytes that developed to morulae was significantly higher for oocytes matured in TCM-199 medium containing cysteamine (14.56 ± 1.06 vs 10.20 ± 0.91) and in SOF medium supplemented with 100 µM of cysteamine (13.97 ± 0.80 vs 10.65 ± 0.40) compared to their respective controls whereas the cysteamine supplementation to the IVM (TCM-199) medium of buffalo oocytes insignificantly increased the cleavage rate when compared with control (33.54 ± 1.54 vs 28.06 ± 2.19) and subsequent development of cleaved embryos to the morula stage (43.40 ± 2.48 vs 36.36 ± 1.65). The cysteamine supplementation to the SOF as IVM medium of buffalo oocytes also insignificantly increased the cleavage rate when compared with control (33.09 ± 1.08 vs 28.99 ± 0.66) and subsequent development of embryos to the morula stage (42.22 ± 3.12 vs 36.73 ± 1.12).

Even though the effect of cysteamine supplementation to IVM was insignificantly higher with regard to cleavage rate and development of early embryos to morulae, the percentage of oocytes that developed to morulae was significantly higher for oocytes matured in TCM-199 medium containing cysteamine (14.56 ± 1.06 vs 10.20 ± 0.91) and in SOF medium supplemented with 100 µM of cysteamine (13.97 ± 0.80 vs 10.65 ± 0.40) compared to their respective controls, which could be attributed to cumulative effect (positive influence) of cysteamine on both the fertilization process and early embryonic development.

The beneficial effect of cysteamine on embryo development might be mediated by an increase in glutathione (GSH) synthesis. Increased oxidative stress appears to be a major factor impairing *in vitro* mammalian embryo development. The oocytes and embryos of buffaloes are more sensitive to oxidative stress because of their higher lipid content which may be a cause of (species-specific) developmental block to embryo development. The effect of

Centralized Embryo Biotechnology Unit, Tamila Nadu Veterinary and Animal Sciences University, Chennai, India, *E-mail: spchigurupati@rediffmail.com, spchigurupati@gmail.com

oxidative stress could be attenuated by efficient antioxidants such as GSH which scavenge reactive oxygen species (ROS). Embryos are capable of synthesizing GSH after the activation of the embryonic genome and the eight-cell block may be correlated to low intracytoplasmic concentration of GSH. Adequate amounts of GSH need to be synthesized during *in vitro* maturation of oocytes in order to support development up to the stage at which embryos acquire the biosynthetic ability.

Keywords: buffaloes, *Bubalus bubalis*, oocytes, TCM-199, synthetic oviductal fluid, SOF, embryonic development

INTRODUCTION

Oocyte maturation can be defined as those events associated with the initiation of germinal vesicle breakdown (GVBD) and completion of first meiotic division (Leibfried-Rutledge *et al*., 1987). Maturation allows the oocyte to express its developmental potential after fertilization and is not merely confined to nuclear events or the ability to be fertilized (Gordon, 2003). The developmental competence of *in vitro* matured oocytes was significantly lower compared to oocytes matured *in vivo*, even though the nuclear maturation rates were similar, due to inappropriate maturation. Therefore maturation media have been supplemented with hormones (Salustri *et al*., 1989), serum (Totey *et al*., 1993), growth factors (Lonergan *et al*., 1996) etc. in order to improve the *in vitro* developmental competence of oocytes.

MATERIALS AND METHODS

The components of various media and consumables used in these experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Nunclon, Denmark, respectively unless and otherwise indicated. All the reagents were of cell culture grade. Milli Q water was autoclave sterilized and used for preparation of different media.

Ovaries from sexually mature buffaloes (*Bubalus bubalis*) were collected irrespective of age, body condition, stage of oestrous cycle and season from Chennai Corporation abattoir and utilized in this study. The ovaries were removed within 30 minutes of slaughter and washed in phosphate buffered saline (PBS) supplemented with 50 µg/ml gentamicin sulphate to remove blood and extraneous material. The washed ovaries were transported at 37^{o}C in a thermos flask in the same media to the laboratory within 30 minutes. The extra-ovarian tissues were trimmed off and the ovaries were washed three times with tap water and five times with PBS to remove blood clots and superficial bacterial contamination. The washed ovaries were kept in a sterile beaker containing PBS supplemented with 50 µg/ml gentamycin until oocyte retrieval. Oocytes were screened under a stereozoom microscope at 10X magnification, washed thrice in 35 mm Petri dishes and graded based on their cumulus mass investment and homogeneity of ooplasm as described by Nandi *et al*. (1998).

IN VITRO MATURATION (IVM) OF OOCYTES

The oocytes were cultured *in vitro* either in synthetic oviductal fluid (SOF) medium or in TCM-

199 supplemented with 10 percent fetal bovine serum (FBS), 100 µM cysteamine, 1.0 µg/ml follicle stimulating hormone (FSH), 0.02 µg/ml luteinizing hormone (LH), 1 µg/ml 17-β estradiol and 50 µg/ml gentamycin. IVM droplets of 100 µl were made, overlaid with sterile mineral oil and equilibrated for 2 h in a CO_2 incubator at 38.5°C and 5 percent CO_2. Only culture grade ('A' and 'B') grade COCs were used for IVM. The COCs were rinsed three times in maturation medium and were transferred to 100 µl of IVM droplets (15 - 20 COCs per droplet). The oocytes were allowed to mature in these droplets at 38.5°C in an atmosphere of 5 percent CO_2 in air for 24 h in a CO_2 incubator.

The maturation of oocytes was assessed based on the expansion of the cumulus mass. The degree of cumulus expansion was assessed by the morphology of the cumulus mass at the end point of *in vitro* maturation as described by Kobayashi *et al*. (1994) and Ravindranatha *et al*. (2002). Oocytes with cumulus expansion of degree 1 and 2 were considered matured. The maturation rate was expressed as percentage of the total number cultured.

Following fertilization (day 0) in BO medium (Brackett and Oliphant, 1975), oocytes were rinsed with TCM-199 and cultured for 10 days in embryo culture medium (TCM-199 with buffalo oviductal epithelial cell (BOEC) clusters) at 38.5°C in an atmosphere of 5 percent CO_2. During the course of culture, half of the embryo culture medium was replaced with fresh embryo culture medium every 48 h.

RESULTS

The percentages of buffalo oocytes that showed cumulus cell expansion in TCM-199 and SOF IVM media are presented in Table 1. Only culture-grade oocytes were used for IVM and the maturation rate was assessed based on the cumulus expansion. Out of 317 oocytes cultured in TCM-199, 292 oocytes showed cumulus expansion with a mean of 92.11 ± 0.60 percent. In SOF IVM medium, out of 296 oocytes cultured, 271 oocytes showed cumulus expansion with a mean of 91.55 ± 0.93 percent. There was no significant (P>0.05) difference between TCM-199 and SOF IVM media with regard to cumulus cell expansion when the COCs were matured *in vitro*. The mean percent of cleavage rate and subsequent development to morulae in buffalo oocytes matured in TCM-199 and SOF media are presented in Table 2. The mean percent of cleavage rate and subsequent development to morulae in buffalo oocytes matured in TCM-199 and SOF media were 28.06 ± 2.19, 10.20 ± 0.91 and 28.99 ± 0.66, 10.65 ± 0.40, respectively. There was no significant difference with regard to cleavage rate and subsequent development to morula when buffalo oocytes were matured in TCM-199 and SOF media

Effect of cysteamine supplementation 100

Table 1. Maturation rates of buffalo oocytes cultured in TCM-199 and SOF media.

IVM medium	No. of oocytes cultured	No. of cumulus expanded oocytes	Maturation rate Mean ± SE	't' value (for arcsine radianse)
TCM - 199	317	292	92.11 ± 0.60	0.079^{NS}
SOF	296	271	91.55 ± 0.93	0.079^{NS}

NS- Not significant (P>0.05).

Table 2. Effect of cysteamine and different maturation media on fertilization and subsequent embryonic development of buffalo oocytes (mean ± SE).

Treatment	Cleavage		Morulae			Blastocysts		
(No. of oocytes)	(n)	% of oocytes	(n)	% of oocytes	% of 2-cell embryos	(n)	% of oocytes	% of 2-cell embryos
TCM-199 Control (196)	55.00 ± 0.98	28.06 [a] ± 2.19	20.00 ± 0.38	10.20 [a] ± 0.91	36.36[a] ± 1.65	6.00 ± 0.24	3.06[a] ± 0.66	10.91 [a] ± 2.07
SOF Control (169)	49.00 ± 0.86	28.99 [a] ± 0.66	18.00 ± 0.33	10.65 [a] ± 0.40	36.73[a] ± 1.12	5.00 ± 0.15	2.96[a] ± 0.71	10.20[a] ± 2.57
TCM-199 with cysteamine (158)	53.00 ± 0.64	33.54 [a] ± 1.54	23.00 ± 0.37	14.56 [b] ± 1.06	43.40[a] ± 2.48	7.00 ± 0.28	4.43[a] ± 1.02	13.21[a] ± 2.74
SOF with cysteamine (136)	45.00 ± 0.39	33.09 [a] ± 1.08	19.00 ± 0.28	13.97 [b] ± 0.80	42.22[a] ± 3.12	5.00 ± 0.28	3.68[a] ± 1.22	11.11[a] ± 4.03

Values within the column with different superscripts differ significantly ($P < 0.05$).

μM on cleavage was presented in Table 2. The percentage of oocytes that developed to morula was significantly higher for oocytes matured in TCM-199 medium containing cysteamine (14.56 ± 1.06 vs 10.20 ± 0.91) and in SOF medium supplemented 100 μM of cysteamine (13.97 ± 0.80 vs 10.65 ± 0.40) compared to their respective controls where as insignificantly higher percentage of cleavage rate and subsequent development of those early embryos to morula were recorded in TCM-199 medium supplemented with 100 μM of Cysteamine (33.54 % ± 1.54 and 43.40 % ± 2.48 respectively) and in SOF medium supplemented 100 μM of cysteamine (33.09 % ± 1.08 and 42.22 % ± 3.12) when compared to TCM -199 (28.06 % ± 2.19 and 36.36 % ± 1.65) and SOF (28.99 % ± 0.66 and 36.73 % ± 1.12) controls respectively.

DISCUSSION

In the present study it was observed that buffalo oocytes showed no significant variation in the percent of cumulus cell expansion when matured in TCM-199 and SOF media. Earlier report (Totey *et al.*,1992) suggested that a significantly higher percent of buffalo oocytes maturation in TCM-199 medium when compared to SOF medium.

The mean percent of cleavage rate and subsequent development to morula in buffalo oocytes matured in TCM-199 and SOF media were 28.06 ± 2.19, 10.20 ± 0.91 and 28.99 ± 0.66, 10.65 ± 0.40 respectively. There was no significant difference with regard to cleavage rate and subsequent development to morula when buffalo oocytes were matured in TCM-199 and SOF media.

The percentage of oocytes that developed to morulae was significantly higher for oocytes matured in TCM-199 medium containing cysteamine (14.56 ± 1.06 vs 10.20 ± 0.91) and in SOF medium supplemented with 100 μM of cysteamine (13.97 ± 0.80 vs 10.65 ± 0.40) compared to their respective controls whereas the cysteamine supplementation to the IVM (TCM-199) medium of buffalo oocytes insignificantly increased the cleavage rate when compared with control (33.54 ± 1.54 vs 28.06 ± 2.19) and subsequent development of embryos to the morula stage (43.40 ± 2.48 vs 36.36 ± 1.65). The cysteamine supplementation to the SOF as IVM medium of buffalo oocytes also insignificantly increased the cleavage rate when compared with control (33.09 ± 1.08 vs 28.99 ± 0.66) and subsequent development of embryos to the morula stage (42.22 ± 3.12 vs 36.73 ± 1.12).

With regard to cleavage rate this finding was contrary to Kundu (2003) but in accordance with the findings of Gasparrini *et al.* (2000 and 2003). who observed no significant effect of cysteamine on maturation and cleavage rate of buffalo oocytes even though the percent of embryos developed to the compact morula and blastocyst stage was significantly ($P < 0.05$) higher for buffalo oocytes matured in medium supplemented with cysteamine. Even though the effect of cysteamine supplementation to IVM was insignificantly higher with regard to cleavage rate and development of early embryos to morulae, the percentage of oocytes that developed to morulae was significantly higher for oocytes matured in TCM-199 medium containing cysteamine (14.56 ± 1.06 vs 10.20 ± 0.91) and in SOF medium supplemented with 100 μM of cysteamine (13.97 ± 0.80 vs 10.65 ± 0.40) compared to their respective controls, which could be attributed to cumulative effect (positive influence) of cysteamine on both the fertilization process and early embryonic development.

Increased oxidative stress appears to be a major factor impairing *in vitro* mammalian embryo

development. The oocytes and embryos of buffaloes are more sensitive to oxidative stress because of their higher lipid content which may be a cause of (species-specific) developmental block to embryo development. The effect of oxidative stress could be attenuated by efficient antioxidants such as glutathione (GSH) which scavenge reactive oxygen species (ROS). Embryos are capable of synthesizing GSH after the activation of the embryonic genome and the eight-cell block may be correlated to low intracytoplasmic concentration of GSH. Adequate amounts of GSH need to be synthesized during *in vitro* maturation of oocytes in order to support development up to the stage at which embryos acquire the biosynthetic ability. GSH also plays an important role during fertilization by contributing to the decondensation of the male pronucleus (Yoshida *et al.,* 1993).

The beneficial effect of cysteamine on embryo development might be mediated by an increase in GSH synthesis (de Matos *et al*., 1997; Gasparrini *et al*., 2000). GSH biosynthesis depended on the availability of cysteine in the extracellular environment. Cysteamine, by reducing cystine to cysteine, elevated the levels of cysteine in the extracellular environment and thereby promoted cysteine uptake and GSH biosynthesis (Gasparrini *et al*., 2003).

The cysteamine supplementation to the medium used for *in vitro* maturation of buffalo oocytes increased glutathione synthesis in the oocyte which participated in sperm decondensation in parallel to oocyte activation and in the transformation of the fertilizing sperm head into the male pronucleus. Cysteamine supplementation might also improve subsequent embryonic development to the morula/ blastocyst stage by protecting cells of early embryos from oxidative stress, promoting amino acid transport, DNA and protein synthesis and reduction of disulphides (Yoshida *et al*., 1993; Lafleur *et al*., 1994 and de Matos *et al*., 1997).

ACKNOWLEDGEMENT

The work was carried out in a scheme on "Establishment of Buffalo ES-Cell Line" funded by Department of Biotechnology, Government of India. The authors are thankful to the funding agency.

REFERENCES

Brackett, B. and G. Oliphant. 1975. Capacitation of rabbit spermatozoa *in vitro. Biol. Reprod.,* **1**: 260-274.

de Matos, D.G., C.C. Furnus and D.F. Moses. 1997. Glutathione synthesis during *in vitro* maturation of bovine oocytes: Role of cumulus cells. *Biol. Reprod.,* **57**: 1420-1425.

Gasparrini, B., G. Neglia, R. Di Palo, G. Campanile and L. Zicarelli. 2000. Effect of cysteamine during in vitro maturation on buffalo embryo development. *Theriogenology,* **54**(9): 1537-1542.

Gasparrini, B., H. Sayoud, G. Neglia, D.G. de Matos, I. Donnay and L. Zicarelli. 2003. Glutathione synthesis during in vitro maturation of buffalo (*Bubalus bubalis*) oocytes: Effects of cysteamine on embryo development. *Theriogenology,* **60**: 943-952.

Gordon, I. 2003. *Laboratory production of cattle embryos: Biotechnology in Agriculture Series, No. 27,* 2nd ed.p p.CABI Publising, UK. 548p.

Kobayashi, K., S. Yamashita and H. Hoshi. 1994.

Influence of epidermal growth factor and transforming growth factor-α on *in vitro* maturation of cumulus cell - enclosed bovine oocytes in a defined medium. *J. Reprod. Fertil.,* **100**: 439-446.

Kundu, A.K. 2003. *In vitro maturation, fertilization, embryo culture and activation treatments of ovine oocytes in different culture systems.* Ph.D. Thesis, Tamil Nadu Veterinary and Animal Sciences University, India.

Lafleur, M.V.M., J.J. Hoorweg, H. Joenje, E.J. Westmijze and J. Retel. 1994. The ambivalent role of glutathione in the protection of DNA against singlet oxygen. *Free Radical Res.*, **21**: 9-17.

Leibfried-Rutledge, M.L., E.S. Crister, W.H. Eyestone and D.L. Northey. 1987. Developmental potential of bovine oocytes matured *in vivo* and *in vitro*. *Biol. Reprod.,* **36**: 376-383.

Lonergan, P., C. Carolan, A.V. Langendonckt, I. Donnay, H. Khatir and P. Mermillod. 1996. Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro*. *Biol. Reprod*., **54**: 1420-1429.

Nandi, S., M.S. Chauhan and P. Palta. 1998. Influence of cumulus cells and sperm concentration on cleavage rate and subsequent embryonic development of buffalo (*Bubalus bubalis*) oocytes matured and fertilized in vitro. *Theriogenology,* **50**(8): 1251-1262.

Ravindranatha, B.M., S. Nandi, P.S.P. Gupta, and P.V. Sarma. 2002. *In vitro* effects of different levels of commercially available PMSG on buffalo oocyte maturation. *Buffalo J*., **18**(1): 101-107.

Salustri, A., M. Yanagishita and V.C. Hascall. 1989. Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone- induced mucification. *J. Biol. Chem*., **264**(23): 13840-13847.

Totey, S.M., G. Singh, M. Taneja, C.H. Pawshe and G.P. Talwar. 1992. *In vitro* maturation, fertilization and development of follicular oocytes from buffalo (*Bubalus bubalis*). *J. Reprod. Fertil*., **95**: 597-607.

Totey, S.M., C.H. Pawshe and G. Singh. 1993. *In vitro* maturation and fertilization of buffalo oocytes (*Bubalus bubalis*): Effects of media, hormones and sera. *Theriogenology,* **39**: 1153-1171.

Yoshida, M., K. Ishigaki, T. Nagai, M. Chikyu and V.G. Pursel. 1993. Glutathione concentration during maturation and after fertilization in pig oocytes relevance to the ability of oocytes to form male pronucleus. *Biol. Reprod.,* **49**: 89-94.