Haryana Vet. **45** (December, 2006), pp 1-6

Review Article

PRESENT STATUS OF *IN-VITRO* EMBRYO PRODUCTION IN BUFFALOES

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In the last several years, there has been an increasing interest in *in-vitro* embryo production (IVEP) in buffaloes because of the low efficiency and poor adoption of AI as well as low efficiency of multiple ovulation and embryo transfer (MOET) (Madan *et al.*, 1996, Gasparrini, 2002). *In-vitro* production technologies not only helps in the production of high genetic merit animals, but also provide an excellent source of embryos for the emerging biotechniques like embryo sexing, cloning, nuclear transfer and transgenesis etc. (Gordon, 1994).

The first buffalo calf "PRATHAM" through *in-vitro* maturation, fertilization, and culture (IVMFC) of buffalo oocytes was reported in 1991 (Madan *et al.*, 1991) and cryopreserved embryo in 1993 (Kasiraj *et al.*, 1993). Since then IVMFC has been successfully used for providing buffalo morula / blastocyst (Totey *et al.*, 1992, 1996, Suzuki *et al.*, 1992, Madan *et al.*, 1994, Narula *et al.*, 1996, Chauhan *et al.*, 1997c, 1997d, 1998a-f, 1999, Nandi *et al.*, 1998) and pregnancies in buffalo (Madan *et al.*, 1994b, Gali *et al.*, 998). The efficiency in terms of transferable embryos and development up to full term has been very low (Madan *et al.*, 1996). In buffalo, a higher rate of maturation (70-94%), fertilization (60-70%) and cleavage (40-50%) with low rates of blastocyst formation (10-15 %) has been reported (Nandi *et al.*, 2002). In cattle, a comparable maturation, fertilization and cleavage rate with a higher proportion of blastocyst development varying from 30-60% has been observed (Holm *et al.*, 1999, Farin *et al.*, 2001). Even though the entire *in- vitro* system establishes in buffalo species has been developed by extrapolating information acquired in more studied species like cattle. The *in-vitro* embryo production of buffalo is considerably less efficient than that of cattle (Zicarelli *et al.*, 1996, Palta and Chauhan, 1998, Nandi *et al.*, 2002). The *in-vitro* culture system employed for performing *in-vitro* fertilized and cleaved embryos up to blastocyst stage are suboptimal and need substantial improvement (Nandi *et al.*, 2002). The present review summarises the *in-vitro* maturation, fertilization, and culture system involved in transferable production in buffalo.

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IN-VITRO MATURATION OF OOCYTES

The washed cumulus oophorus complexes (COCs) are generally cultured in 50 µl droplets (15-20 oocytes / droplet) of maturation medium in a 35 mm sterile petridish. The droplets are covered with warm non-toxic paraffin oil and placed in CO_2 incubator at 38.5^{0}C with 5% CO_2 in air and relative humidity for 24 hours.

Buffalo oocytes are mostly cultured in complex medium such as TCM-199 (Singh *et al.*, 1989, Totey *et al.* 1991, 1992, 1993, Madan *et al.*, 1994a, Dhanda *et al.*, 1996, Das *et al.*, 1996, Chauhan *et al.*, 1997a), Ham's F-10 medium (Totey *et al.*, 1993), minimum essential medium and way mouth medium (Ravindranatha *et al.*, 2001) for 24 h under standard conditions. Supplementation of IVM culture medium with fetal calf serum (FCS) (Totey *et al.*, 1996, Chauhan *et al.*, 1999), proestrus buffalo serum (PrBS) (Samad *et al.*, 1998), oestrus buffalo serum (OBS) (Singh and Majumdar 1992, Totey *et al.*, 1992), oestrus cow serum (OCS) (Samad *et al.*, 1998), steer serum (SS) (Chauhan *et al.*, 1998a, Nandi *et al.*, 2001b) or super ovulated buffalo serum (SBS) (Chauhan *et al.*, 1998a) at 10-20% oxygen tension is necessary for achieving

high nuclear and cytoplasmic maturation rates of 60-80%.

Hormones like human chorionic gonadotrophin (HCG) (Chuangsoongneon and Kamonpatana, 1991), follicle stimulating hormone (FSH) (Chauhan *et al.*, 1996), pregnant mare serum gonadotropin (PMSG) (Gupta *et al.*, 2001), luteinizing hormone (LH) and estradiol either alone or in combination (Totey *et al.*, 1992, Nandi *et al.*, 2002) have been used to induce higher maturation rates ranging from 60-85%. Various growth factors such as epidermal growth factor (EGF) (Chauhan *et al.*, 1999), EGF plus fibroblast growth factor (Gupta *et al.*, 2002), insulin like growth factor-I (IGF-I) (Pawshe *et al.*, 1998), insulin like growth factor –II (IGF-II) (Chauhan *et al.*, 1998c) in oocytes culture media have shown to increase cumulus expansion, nuclear maturation (80-90%) and post fertilization cleavage rates. In addition, defined and semi defined media can be successfully used for IVM of buffalo oocytes (Abdoon *et al.*, 2001, Gupta *et al.*, 2002). To develop cost effective maturation media, attempts have been made to replace the basic medium partially (maturation rate 65%) or completely (maturation rate 100%) with follicular fluid (Chauhan *et al.*, 1997c, Gupta *et al.*, 2001).

IN-VITRO FERTILIZATION OF OOCYTES

In-vitro matured buffalo oocytes are co-incubated with frozen-thawed *in-vitro* capacitated spermatozoa in Tyrodes modified medium (TALP) (Totey *et al.*, 1996) or Brackett and Oliphant (BO) medium (Nandi *et al.*, 1998) for fertilization. An *in-vitro* fertilization rate of 60-80% has been reported in both the media. *In-vitro* capacitation of buffalo spermatozoa is carried out by incubating motile spermatozoa separated by swim up (Nandi *et al.*, 1998) or Percoll gradient technique (Totey *et al.*, 1996). Commonly used sperm motility enhancers include caffeine (Chauhan *et al.*, 1998a, Nandi *et al.*, 1998), theophylline (Jainudeen *et al.*, 1993, Chauhan *et al.*, 1998f), pentoxifyline (Ramesha *et al.*, 2002) and a mixture of penicillamine, hypotaurine and epinephrine (PHE) (Totey *et al.*, 1996). A fertilization rate of 55% and 40% were reported with caffeine and theophylline, respectively. Considerable variations have been reported among different buffalo bulls in terms of the ability of their spermatozoa to fertilize oocytes *in-vitro* (Totey *et al.*, 1996, Chauhan *et al.*, 1998d, Yadav *et al.*, 2001). Different authors have reported different sperm concentration and sperm oocyte co-incubation period optimum for buffalo IVF. A sperm concentration of 4-5 millions with a co-incubation period of 20-24 hours resulted in 60-75% fertilization rate (Totey *et al.*, 1996, Nandi *et al.*, 1998). Attempts have also been made to study the effect of cumulus cells on sperm penetration and subsequent fertilization rate (72%) (Nandi *et al.*, 1998).

IN-VITRO CULTURE OF EMBRYOS

A variety of embryo culture systems have been employed for *in-vitro* embryo production system. Among these, major systems of *in-vitro* culture of embryo as reported by Walker *et al.*, (1996) are (i) transferring embryos to ligated oviduct of surrogate recipient (ii) culturing them *in-vitro* with somatic cells (oviduct epithelial cells, granulosa cells, Vero-cells) in a complex medium like TCM-199 (iii) culturing such embryos in simple medium like synthetic oviductal fluid (SOF) without the support of somatic cells. Attempts to culture *in- vitro* produced buffalo embryos *in -vivo* in rabbit oviduct (Ahluwalia and Majumdar, 1992) and in the amniotic cavity of developing chick embryo (Ocampo *et al.*, 2001) were found with limited success.

Factors affecting IVC

Effect of media: With the exception of a very few studies in which defined media like synthetic oviductal fluid (SOF) or modified synthetic oviductal fluid (mSOF) has been used for culture of buffalo embryos (Totey *et al.*, 1996, Abdoon *et al.*, 2001), most investigator have used complex media containing serum and somatic cells (Chauhan *et al.*, 1998a-f, Nandi *et al.*, 2000, 2001a). Due to variability in composition and risk of infection in complex media, embryo culture in serum free defined media has been recommended (Nandi *et al.*, 2002).

The most common medium for *in-vitro* culture is SOF or a modified form of SOF

(mSOF). Synthetic oviductal fluid containing 0.3% BSA with different additives improved blastocyst development rate up to 45-50% in IVC of cattle embryos (Holm *et al.*, 1999). A higher blastocyst rate (14% Vs 7%) was obtained when buffalo embryos were cultured in SOF as compared with TCM-199 having 10% FCS in co-culture (Gasparrini, 2002). In contrary, a higher blastocyst yield of 24% Vs. 13% was observed in TCM-199 supplemented with steer serum and oviductal cell co-culture than in mSOF (Nandi *et al.*, 2002). In our laboratory, *in-vitro* culture of buffalo embryos in modified synthetic oviductal fluid (mSOF) resulted in an average cleavage rate of 42% with concomitant development of 42% morula stage (Kalleshwarappa, 2002) and 48% transferable embryo production (Arathy, 2003). Supplementation of gelatin increased the cleavage of eggs up to the 8-16 cell-stage embryo, but did not significantly enhance the rate of development to the morula/blastocyst (Arathy *et al.*, 2004).

Development of blastocyst stage in case of bovine was found to be higher in Charles Rosenkrans medium1 (CR1aa) (46%) when compared to Charles Rosenkrans medium2 (CR2), bovine embryo culture medium (BECM-g) and tissue culture medium (TCM-199) (Dogan *et al.*, 2002) indicating that initial development of embryo in simple media is better than complex media. New concepts of *in-vitro* embryo culture in cattle and human can be applied to buffalo by adopting sequential media system (Gardner and Lane, 1998, Chauhan *et al.*, 1999), autocrine and paracrine factors and regulators of energy metabolism (Thompson, 2000).

Supplementation with protein sources: Bovine serum albumin (BSA) and serum from different sources are the most commonly used protein supplements of IVC media. In addition to protein, serum contains amino acids, hormones, growth factors, cytokines, vitamins and many other substances, which are found to be supportive of embryo development. However, some of the reports suggested that presence of serum during early cleavage inhibits further development and in later stages favors development (Pinyopummintr *et al.*, 1994). Addition of fetal calf serum (FCS) to SOF containing BSA promotes blastocyst development (28%) with lower embryo viability (Yoshika *et al.*, 1997, Thompson *et al.*, 2001). But addition of FCS on day 6 of post insemination to SOF droplets significantly improved blastocysts yield (38%) with higher blastocysts cell number (Carolan *et al.*, 1995). Oestrus cow serum (Samad *et al.*, 1998) and steer serum (Nandi *et al.*, 2002) were found to support embryonic development. Kuran *et al.* (2002) are of pinion that supplementation of 0.04% BSA to the SOF improved blastocyst number yield (50%), total cell and hatching indices of embryo.

Effect of co-culture: The introduction of co-culture system in the mid 1980 helped to alleviate the *in-vitro* induced arrest at 8-16 stage and tabulated development to the blastocyst stage. Bovine morulae and blastocyst can be obtained from *in-vitro* matured and fertilized oocytes by co-culturing them with various somatic cells (Eyestone and First, 1986) or culturing them in oviductal cell conditioned medium (Eyestone and First, 1989). Cell types, other than oviductal cells were successfully employed for supporting high rates of *in-vitro* embryonic development including buffalo rat liver cells (BRL) (Reed *et al.*, 1996) and Vero cells (Ramdoss, 1991)

Buffalo blastocyst production has been achieved by culturing embryos in complex media supplemented with serum and somatic cell co-culture (cumulus cells and / or oviduct epithelial cells) (Madan *et al.*, 1994a, Chauhan *et al.*, 1998a-f, Nandi *et al.*, 1998, 2002). It has been reported that heterologus or homologus oviductal cells can also be used to support embryo development up to blastocyst stage in buffalo (Yadav *et al., 2000*). In buffalo BRL cells and Vero cells have been successfully used for blastocyst production (Gasparrini, 2002). Higher blastocyst yield was noted in droplets containing lower number of motile oviductal epithelial cells when compared to those with higher number of oviductal cells (Majumdar and Sharma, 2001).

Supplementation of growth factors: The culture medium containing insulin like growth factor-I (IGF-1), (Narula *et al.*, 1996) and /or growth hormone (GH) (Moreira *et al.*, 2002) were found to be supportive of *in-vitro* embryo development in cattle (30-40%) as well as in buffalo (10-15%). Supplementation of embryo

culture medium with insulin (Chauhan *et al.*, 1998b) and a combination of BSA, EGF (epidermal growth factor) and ITS (insulin-transferrin-selenium) (Shamsuddin *et al.*, 1994, Palasz and Thundathil, 1998) improved development and viability of embryos.

Effect of other additives: A mixture of vitamins and amino acid supplementation noticeably improved *in-vitro* development of cattle embryos. Temporal and differential effect of amino acids during embryo development was reported by Steves and Gardner (1999). A new concept is that, the regulators of energy metabolism compounds such as ethylene diamine tetra acetic acid (EDTA) and 2, 4 dinitro phenol have been shown in ruminants to improve quality of developing embryos (Thompson, 2000).

CULTURE OF EMBRYO UP TO HATCHING STAGE

Progressive development of embryos can be assessed for determining viability. Studies in cattle have shown that the embryo that undergoes cleavage earlier has a greater chance of developing to morulae/blastocyst (Plante and King, 1992). Hatching has also been employed as a measure of the ability of embryo to progress normally after various treatment or manipulation such as freezing and thawing (Chauhan *et al.*, 1998e). A superficial evaluation of the quality of IVMFC derived buffalo embryos can be done by observing the stage of development in relation to time (Chauhan *et al.*, 1998e).

REFERENCES

Abdoon, A.S.S., Kandil, O.M., Otoc, T. and Suzuki, T. (2001). Influence of oocyte quality, culture media and gonadotrophins on cleavage rate and development in *in- vitro* fertilized buffalo embryos. *Anim. Reprod. Sci.* **64:** 215-223.

Ahluwalia, R.S. and Majumdar, A.C. (1992). *In vivo* culture in rabbit oviduct of *in vitro* fertilized bubaline oocytes. *Indian J. Anim. Sci.* **62:** 528-531.

Arathy, D.S. (2003). Studies on development of *in- vitro* generated buffalo embryos in culture. M.V.Sc. thesis, Indian Veterinary Research Institute, Izatnagar, India.

Arathy, D.S., Ashish, S., Sharma, G.T., Majumdar, A.C. and Chauhan, M.S. (2004). Effect of specific gravity of culture medium on in vitro embryo development in buffalo. *Reprod. Feritl. Dev.***16:** 253

Carolan, C., Lonergan, A., Van Langendonekt, A. and Mermillod, P. (1995). Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization *in vitro*. *Theriogenol.* **43:** 1115-1128.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R..S. and Madan, M.L. (1999). Effect of EGF on cumulus expansion, meiotic maturation and development of buffalo oocytes *in- vitro*. *Vet. Rec.* **144:** 266-267.

Chauhan, M.S., Katiyar, P.K., Madan, M.L., Singla, S.K and Manik, R.S. (1996). Influence of follicle stimulating hormone on *in-vitro* maturation and cleavage of buffalo (*Bubalus bubalis*) oocytes after *in-vitro* fertilization. *Theriogenol.* **45:** 243.

Chauhan, M.S., Katiyar, P.K., Singla, S.K., Manik, R.S and Madan, M.L., (1997a). Production of buffalo calves through *in- vitro* fertilization. *Indian J. Anim. Sci.* **67:** 306-308.

Chauhan, M.S., Manik, R.S., Singla, S.K., Katiyar, P.K. and Madan, M.L., (1997b). Effect of insulin on in in-vitro development of *in-vitro* produced embryos in co-culture system. *Indian J. Anim. Prod. Manag.* **13:** 19-24.

Chauhan, M.S., Palta, P., Das, S.K., Katiyar, P.K. and Madan, M.L. (1997c). Replacement of serum and hormone additives with follicular fluid in IVM medium: effect of maturation, fertilization and subsequent development of buffalo oocytes *in-vitro*. *Theriogenol.* **48:** 461-469.

Chauhan, M.S., Singla, S.K., Manik, R.S and Madan, M.L. (1997d). Increased capacitation of buffalo sperm by heparin as confirmed by electron microscopy and *in-vitro* fertilization. *Indian J. Exp. Biol.* **35:** 1038-1043.

Chauhan, M. S., Singla, S.K., Palta, P. and Manik, M.L. (1998a). *In-vitro* maturation and fertilization, and subsequent development of buffalo *(Bubalus bubalis)* embryos: effects of oocyte quality and type of serum. *Repro. Fertl. Dev.* **10:** 173-177.

Chauhan, M.S., Palta, P., Das, S.K., Katiyar, P.K. and Tomer, O.S. (1998b). Effect of culture conditions on the hatching ability of *in vitro* produced buffalo (*Bubalus bubalis*) oocytes *in-vitro*. *Vet. Rec.* **142:** 169-171.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S. and Tomer, O.S. (1998c). IGF-II stimulation of *in vitro* maturation, *in-vitro* fertilization and subsequent development of buffalo (*Bubalus bubalis*) oocytes *in-vitro*. *Vet. Rec.* **142:** 727-728.

Chauhan, M.S., Singla, S.K., Palta, P. and Manik, R.S and Madan, M.L. (1998d). Individual variation among buffalo bulls in fertilization and subsequent embryonic development *in-vitro*. *Indian J. Anim. Sci.* **68**: 454-456.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S and Tomer, O.S. (1998e). Development of *in vitro* produced buffalo (*Bubalus bubalis*) embryos in relation to time. *Asian Aust. J. Anim. Sci.* **11:** 398-403.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S. and Madan, M.L. (1998f). Influence of theophylline on cleavage rate and embryonic development following

in-vitro fertilization of buffalo oocytes. *Indian. J. Anim. Sci.* **68:** 920-922.

Chuangsoongneon, U. and Kamonpatana, M. (1991). Oocyte maturation, *in-vitro* fertilization and culture system for developing preimplantation Swamp buffalo embryos using frozen-thawed semen. *Buffalo J.* **17:** 189-198.

Das, S.K., Chauhan, M.S., Palta, P., Katiyar, P.K. and Madan, M.L.(1996). Replacement of fetal bovine serum and FSH with buffalo follicular fluid in *in-vitro* maturation of buffalo oocytes. *Theriogenol.* **45:** 245

Dhanda, O.P., Saini, M.S., Lohan, I.S. and Malik, R.K.(1996). Potential use of rabbit perotonial fluid for *in-vitro* production of caprine embryos. In: Advances, Human and Animal Reproduction (Ed.) Mathur, Jivaji University, Gwalior, India.

Dogan, I., Sagirkaya, H.,Soylu, M.K., Nur, Z. and Yerikaya, H. (2002). Development of *in-vitro* produced bovine embryo in four different culture media. *Indian J. Anim. Sci.* **72:** 227-229.

Eyestone, W.H. and First, N. L. (1986). A study of 8 to 16 cell developmental block in bovine embryos cultured *in-vitro*. *Theriogenol.* **25**: 152.

Eyestone, W.H. and First, N.L. (1989). Co- cultures of early bovine embryos with oviductal tissue or in conditioned medium. *J. Repro. Fertl.* **85:** 715-720

Farin, P.W., Croseir, A.E. and Farin, C.E. (2001). Influence of *in-vitro* system on embryo survival and fetal development in cattle. *Theriogenol.* **55:** 151-170.

Galli, C., Duchi, R., Crotti, G. and Lazzari, G. (1998). Embryo production by ovum pick up *in vitro* embryo technology. *Theriogenol.* **49:** 400.

Gardner, G.K. and Lane, M. (1998). Culture of viable human blastocysts in defined sequential serum-free media, *Human Reprod.* **13:** 101-102.

Gasparrini, B., Naglia, G., Palo, Di R., Companile, G. and Zicarelli, L. (2001). Effect of cysteamine during *in-vitro* maturation of buffalo embryo development. *Theriogenol.* **54:** 1537-1542.

Gassparrini, B. (2002). In-vitro embryo production in buffalo species: State of the art. *Theriogenol.* **57:** 237-256.

Gordon, I. (1994).Aspiration techniques:old and new. In "Laboratory Production of Cattle Embryo". (Ed. I. Gordon) pp71-72 (CAB International, Wallington, U. K.).

Gupta, P.S.P., Nandi, S., Ravindranatha, B.N. and Sarma, P.V. (2001). Effect of buffalo follicular fluid alone or in combination with M199 and PMSG on buffalo oocyte maturation. *Asian Aust. J. Anim. Sci.* **14:** 693-696.

Gupta, P.S.P., Nandi, S., Ravindranatha, B.N. and Sarma, P.V. (2002). *In-vitro* maturaion of buffalo oocytes with epidermal growth factor and fibroblast growth factor. *Indian J. Anim. Sci.* **72**: 20-23.

Holm, P., Booth, P.J., Schmidt, M. H., Greve, T. and Callesen, H. (1999). High bovine blastocysts development in a static *in-vitro* production system using SOFaa medium supplemented with sodium citrate and myoinositol with or without serum proteins. *Theriogenol.* **52:** 683-700.

Jainudeen, M.R., Takahashi, Y., Nihayah, M. and Kanagawa, H. (1993). *In-vitro* maturation and fertilization of swamp buffalo (*Bubalus bubalis*) oocytes. *Anim. Reprod. Sci.* **31:** 205-212.

Kalleshwarappa, G.M. (2002). Studies on the secretory proteins in- vitro matured *in-vitro* fertilized buffalo embryos. Ph. D. thesis, Deemed University, IVRI, Izatnagar, India.

Kasiraj, R. Misra, A.K., Mutha Rao, M., Jaiswal, R.S. and Rangareddy, N. (1993).Successful culmination of pregnancy and live birth following the transfer of frozen embryo. *Theriogenol.* **39:** 1187-1192.

Kuran, M., Robinson, J.J., Brown, D.S. and McEvoy, T.G. (2002). Development, amino acid utilization and cell allocation in bovine embryos after *in-vitro* production in contrasting culture systems. *Reprod.* **124:**155-165.

Madan, M.L., Singla, S.K., Jailkhani, S., and Ambrose, J.D. (1991). *In-vitro* fertilization and birth of first ever IVF buffalo calf. In: Proc. Third World Buffalo Congress, Varna, Bulgaria.**7**:11-17.

Madan, M.L., Singla, S.K., Chauhan, M.S. and Manik, R.S. (1994a). *In-vitro* production and transfer of embryos in buffaloes. *Theriogenol.* **41:** 139-143.

Madan, M.L., Chauhan, M.S., Singla, S.K., and Manik, R.S. (1994b). Pregnancies established from water buffalo (*Bubalus Bubalis*) blastocyst derived from *in-vitro* matured, *in-vitro* fertilized and co-cultured with cumulus and oviductal cells. *Theriogenol.* **42:** 591-600.

Madan, M.L., Das, S.K. and Palta, P. (1996). Application of reproductive technology to buffaloes. *Anim. Reprod. Sci.* **42:** 299-306

Majumdar, A.C. and Sharma, G.T. (2001). Does embryo needs constant rotation for formation of blastocyst? XII[th] Annual Conf. of Society of Animal Physiologists of India (SAPI).

Moreira, F, Paula-Lopes, F.F., Hansen, P.J., Badinga, L. and Thatcher, W.W. (2002). Effects of growth hormone and insulin-like growth factor-I on development of invitro derived bovine embryo. *Theriogenol.* **57**: 895-907

Nandi, S., Chauhan, M.S. and Palta, P. (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*. *Theriogenol.* **50:** 1251-1262.

Nandi, S., Chauhan, M. S. and Palta, P. (2000). Effect of a corpus luteum in the recovery and developmental potential of buffalo oocytes. *Vet. Rec.*, **147:** 580-581.

Nandi, S., Chauhan, M.S. and Palta, P. (2001a). Effect of environmental temperature on quality and developmental competence *in-vitro* of buffalo oocytes. *Vet Rec* **148:** 278-279.

Nandi, S., Gupta, P.S.P., Ravindranatha, B.N. and Sarma, P.V. (2001b). Influence of different levels of steer serum on production of fertilizable buffalo oocyte *in-vitro*. *Vet. Rec.*, **149:** 124-125.

Nandi, S, Raghu, H.M., Ravindranatha, B.M. and Chauhan, M.S. (2002). Production of buffalo (Bubalus bulalis) Embryos *in-vitro*: premises and Promises *Reprod. Domestic Anim.* **37**: 65-74.

Narula, A., Taneja, M. and Totey, S.M. (1996). Morphological development, cell number and allocation of cell to trophectoderm and inner cell mass of *in-vitro* fertilized and parthenogenetically developed buffalo embryos: the effect of IGFI. *Mol. Reprod. Dev.* **44**: 343-351.

Ocampo, L.C., Mamuad, F.V., Mori, T., Shimizu, H., Ocampo, M.B. (2001). In vitro production of pre-implantation buffalo embryos. *Buffalo J.* **17:** 145-154.

Palasz, A.T. and Thundathil, J. (1998). Effect of volume of culture medium and embryo density on *in- vitro* development of bovine embryos. *Theriogenol.* **49:** Abstr 212.

Palta, P. and Chauhan, M.S. (1998). Laboratory production of buffalo (*Bubalis bubalis*) embryos. *Reprod. Fertl. Dev.* **10:** 379-391.

Pawshe, C.H., Appa, Rao, K.P.C.A. And Totey, S.M. (1998). Effect of insulin-like growth factor I and its interaction with gonadotrophins on *in vitro* maturation and embryonic development, cell proliferation and biosynthetic activity of cumulus-oocyte complexes and granulose cell sin buffalo. *Mol. Reprod. Dev.* **49:** 277-289.

Pinyopummintr, T. and Bavister, B.D. (1994). Development of bovine embryos in a cell-free culture medium. Effects of type of serum,timing of its inclusion and heat inactivation. *Theriogenol.* **41:** 1241-1249.

Ramdoss, S. (1991). Studies on the *in- vitro* fertilization of buffalo follicular oocytes from ovaries obtained from abattoir. M.V.Sc. thesis, Indian Veterinary Research Institute, Izatnagar, India.

Ramesha, K.P., Balakrishnan, M.M., Murthy, L.K. and Balakrishnan, C.R. (2002). Additive effects of pentoxyfyline and heparin on buffalo sperm motility and fertilization of oocytes matured in culture. *Buffalo J.* **16:** 63-71.

Ravindranatha, B.M., Nandi, S., Gupta, P.S.P. and Sarma, P.V. (2001).Comparision of different media on maturation of buffalo oocyte maturation. *Indian J. Anim. Sci.* **71**: 841-843.

Reed, W.A., Suh, T., Bunch,T.P. and White, K.L. (1996).Culture of in vitro fertilized bovine embryos with bovine oviductal epithelial cells,Buffalo rat liver(BRL) cell or BRL-cell conditioned medium. *Theriogenol.* **45:** 439-449.

Samad, H.A., Khan, I.Q., Rehmanb, N.U. and Ahmed, N (1998) The recovery, *in vitro* maturation and fertilization of Nilli-Ravi buffalo follicular oocytes. *Asian-Aust. J. Anim. Sci.* **11:** 491-497.

Shamsuddin, M., Larrson B., Gustafasson H and Rodriquez Martinez, H. (1994). A serum free, cell free culture system for development of bovine one-cell embryos up to blastocysts stage with improved viability. *Theriogenol.* **41:** 1033-1043.

Singh, G., Panagaonkar, G.R., Matharaoo, J.S., Singh, P. and Singh, M. (2002). Dye exclusion technique to assess the viability of pre-implantation bovine embryo based on a murine model. *Indian J. Anim. Reprod.* **23:** 12-17.

Singh, G., Totey, S.M and Talwar, C.P.(1989). *In-vitro* fertilization of buffalo oocytes matured *in-vitro*. *Theriogenol.* **31:** 225(Abstr).

Singh, R. and Majumdar, A.C. (1992). Chronological changes of buffalo follicular oocytes maturation in-vitro. *Indian J. Anim. Sci.* **62:** 205-209.

Steeves, T E. and Gardner, D.K. (1999). Temporal and differential effects of amino acid on bovine embryo development in culture. *Biol. Reprod.* **61:** 731-740.

Suzuki, T., Singla, S.K., Sujata, J.M and Madan, M.L. (1992). *In-vitro* fertilization of water buffalo follicular oocytes and their ability to cleave *in vitro*. *Theriogenol.* **38:** 1187-1194.

Thompson, J.G. (2000). *In-vitro* culture and embryo metabolism of cattle sheep embryos- a decade of achievement. *Anim. Reprod. Sci.* **60:** 263-275.

Thompson, J.G., Allen, N.W., McGowan, L.T. Bell, A.C. S., Lambert, M.G. and Tervit, H..R. (1998). Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development *in-vitro* and following transfer. *Theriogenol.* **49:** 1239-1249.

Totey, S.M., Daliri, M., Appa Rao, K.B.C., Pawshe, C.H., Taneja, M. and Chiller, R.S. (1996). Differential cleavage and developmental rates and their correlation with cell numbers and sex ratios in buffalo embryos generated *in-vitro*. *Theriogenol.* **45:** 521-533.

Totey, S.M., Pawshe, C.H. and Singh, G.P. (1993). Effect of bull and heparin and sperm conc. on *in-vitro* fertilization of buffalo (*Bubalus bubalis)* oocytes matured in vitro. *Theriogenol.* **39:** 887-898.

Totey, S.M., Singh, G.P, Gurpreet, Taneja, M., Pwshe, C.H. and Talwar, G.P. (1991). *In-vitro* maturation and fertilization of follicular oocytes from buffalo. *Theriogenol.* **35** :284.

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

Walker, S.K., Hill, J.L., Kleeman, D.O. and Nancarrow, C.D. (1996). Development of ovbine embruos in synthetic oviductal fluid containing amino acids at oviductal concentrations. *Biol. Reprod.* **55:** 703-708

Yadav, P.S., Hooda, O.K., Saini, A. and Jindal, S.K. (2001). *In-vitro* cleavage and development of buffalo oocytes with semen from buffalo bulls. *Indian. J. Anim. Sci.* **71:** 752-754.

Yadav, P.S., Khanna, S., Hooda, O.K. and Sethi, R.K. (2000). Effect of oviductal cell co-culture on claeavage and development of buffalo IVF embryos. *Asian-Aust. J. Anim. Sci.* **13:** 894-896.

Yoshioka, K., Othman, A.M., Taniguchi, T., Yamanaka, H. and Sckikawa K. (1997). Differential patterns of blastulation in bovine morulae cultured in synthetic oviduct fluid medium containing FCS or BSA. *Theriogenol.* **48:** 997-1006.

Zicarelli, L., Boni, R., Campanile, G., Palo, R.D., Esposito, L., Roviello, S., Varriccho, E. and Langella, M. (1996). Super ovulation, ovum pick up and *in-vitro* embryo production in Mediterranean buffaloes bred in Italy. *Bulg. J. Agri. Sci.* **2:** 115-120.