Reproductive Biotechnologies for Improvement of Buffalo: The Current Status

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ABSTRACT : Reproductive biotechnologies continue to be developed for genetic improvement of both river and swamp buffalo. Although artificial insemination using frozen semen emerged some decades back, there are still considerable limitations. The major problem appears to be the lack of efficient methods for estrus detection and timely insemination. Controlled breeding experiments in the buffalo had been limited and similar to those applied in cattle. Studies on multiple ovulation and embryo transfer are essentially a replica of those in cattle, however with inherent problems such as lower number of primordial follicles on the buffalo ovary, poor fertility and seasonality of reproduction, lower population of antral follicles at all stages of the estrous cycle, poor endocrine status and a high incidence of deep atresia in ovarian follicles, the response in terms of transferable embryo recovery has remained low with 0.51 to 3.0 per donor and pregnancy rates between 15 to 30%. *In vitro* production of buffalo embryos is a valid alternative to recovery of embryos by superovulation. This aspect received considerable attention during the past decade, however the proportion of embryos that develops to the blastocyst stage is still around 25-30% and hence the *in vitro* culture procedures need substantial improvement. Embryo cryo-preservation procedures for direct transfer post thaw need to be developed for bubaline embryos. Nuclear transfer and embryo cloning is a technique that has received attention in various species during recent years and can be of immense value in buffaloes as they have a low rate of embryo recoveries by both *in vitro* and *in vivo* procedures. Gender pre-selection, genome analysis, gene mapping and gene transfer are a few of the techniques that have been studied to a limited extent during recent years and are likely to be included in future studies on buffaloes. Very recently, reproductive biotechnologies have been applied to feral buffaloes as well, but the results obtained so far are modest. When fully exploited they can play an important role in the preservation of endangered species. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 7 :1071-1086)***

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INTRODUCTION

Buffalo (*Bubalus bubalis*) constitute the mainstay of the dairy industry in middle and southern Asia. Buffalo has the potential superiority over cattle under the harsh climate of tropics, so should be genetically exploited. Specialized reproductive technologies continue to provide new opportunities for consistent improvement and safety of products from economically valuable food animals and safeguarding rare species. This has been achieved by dissemination of elite stock through artificial insemination and it is expected that the livestock industry will be dominated with progeny tested bulls screened for genetic markers of economic traits. Sex-selected semen (Johnson, 1992; Windsor et al., 1993; Sood, 1997) embryo sexing (Sood, 1997; Appa Rao and Totey, 1999) and cloning (Singla, 1996; Parnpai et al., 2000) are expected to produce animals of preferred genotype and gender. Animal biotechnology has shown its impact on genetic selection, animal health, production and efficiency, animal nutrition and reproduction, and niche markets for specialized animal products (Heap and Spencer, 1998).

The biotechniques developed so far have been used for achieving various goals in animal production such as efficient utilization of germ cell production in males and females, worldwide exchange of genetic material through use of frozen semen and embryos (Nilmann, 1991; Ranjan and Pathak, 1993; Mahmoudzadeh et al., 1996; Cruz, 1998). Laboratory production of embryos allows mass scale production of embryos for practical and scientific purposes as well as for the exploitation of oocytes from donors (Drost et al., 1983; Totey et al., 1991; Madan et al., 1994b; Misra et al., 1994; Cruz, 1998; Galli et al., 1998). Improvement of genetic progress could be hastened by integrated breeding programs based on biotechnological procedures like sexing, cloning and *in vitro* production of embryos. New products like novel proteins in milk of farm animals can be produced by gene transfer (Brink et al., 2000). Gene transfer could be helpful for pharmaceutical protein synthesis at comparatively lower costs, improving animal health and introduction of specific traits (Bremel, 1996). Estimation of breeding values, an accurate genetic progress and eradication of genetic disorders and genetically determined diseases could be made possible by genome analysis and gene mapping (Arvindakshan et al., 1997).

Despite various attempts for effective utilization of

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these techniques the improvement in reproductive efficiency for buffaloes has remained marginal and the techniques are yet to become commercially viable. In this paper the focus on reproductive biotechniques and their use for genetic improvement of buffaloes are limited to artificial insemination, cryopreservation of germ cells and embryos, ovum pick up from ovaries *in situ*, and *in vitro* maturation, fertilization and culture. Prospective areas of gender preselection, cloning, genome analysis, gene mapping and gene transfer are also addressed.

ARTIFICIAL INSEMINATION (AI)

The earliest application of biotechnology for improved reproduction and thereby speeding up the rate of genetic improvement through the male path was artificial insemination. The AI technique makes efficient use of semen (through dilution and appropriate doses) over many females, use of semen of males having superior genotype and also increasing the rate of conception through proper estrus detection, reproductive organ management and medication. In India the first buffalo calf was born through AI in 1943 (Ranjan and Pathak, 1993). Since then, many more have born and it has expanded in rural areas with acceptability by farmers. However, much is still needed to cover the entire buffalo population. After cryopreservation of semen became viable in buffaloes in 1972, the extenders, cryoprotectant levels and freezing procedures have become standardized for good fertility and AI has since been used globally.

Semen dilution

Conventional extenders like egg yolk citrate or milk used for cryopreservation of bull spermatozoa are considered sub-optimal for buffalo semen. The most suitable extender for buffalo semen is tris-yolk-glycerol with caffeine citric acid and raffinose (Dhami et al., 1994). Although Kumar et al. (1993) suggest cow milk as a good dilutor for preserving buffalo semen, Akhtar et al., 1990 considered tris to be superior over milk. Tes-tris-yolk-glycerol and mes-tris-yolk-glycerol have been found equally good to tris dilutors (Oba et al., 1994; Chachur et al., 1997). The acceptable level of glycerol is 6-7% and an equilibration time of 2-6 h is considered optimum. Likewise the widely acceptable level of egg yolk is 20%. A slow cooling procedure (0.2-0.4°C/min) is recommended during pre-freezing processing of buffalo semen (Sansone et al., 2000). The freezing of semen is usually done by suspending the semen straws in horizontal position 1-4 cm above liquid nitrogen for 10-20 min, after which they are immersed into liquid nitrogen at -196°C. A step-wise freezing procedure using a programmable freezer is reported to yield better post-thaw recoveries (Del Sorbo et al., 1995), however the freezability of buffalo bull semen is considered to be poor in general. The poor freezability of buffalo spermatozoa compared to bull spermatozoa is considered to be due to inherently fragile plasma membrane of buffalo spermatozoa and considered to be due to the low membrane phospholipid content and its loss during freezing-thawing (Sansone et al., 2000). Additives like cysteine hydrochloride, EDTA or membrane stabilizers like chlorpromazine hydrochloride are used to minimize enzyme leakage and acrosomal damage during freezing. The buffalo bull semen differs from bull semen in respect of several constituents. Compared to bull semen it has high contents of calcium, chloride, acid soluble phosphorus, and alkaline phosphatase, and lower total electrolytes, ascorbic acid and seminal protein. These altered ratios of constituents are considered responsible for poor buffering capacity and poor preservability of buffalo bull semen (Agarwal and Tomar, 1998). Sephadex and Sephadex ion-exchange filtration is reported to improve the quality and freezability of extended, low quality buffalo bull semen (Ahmad et al., 2003). Thawing of frozen semen is done in a water bath at 40°C for 30 seconds (Dhami et al., 1994, Vale, 1997) although it can be done at the higher temperature of 50°C for 15 seconds.

Conception rates

There has been much concern about semen preservation and establishment of global gene pools and development of an effective system of AI service delivery (Cruz, 1998). Buffaloes in the Indian sub-continent comprise many breeds having the best milk production capacity (Murrah and Nili-Ravi) and fat content (Surti, Bhadavari and Jaffarabadi). This gene pool is required to be preferentially selected and propagated.

The major constraints in the effective utilization of AI in buffaloes include seasonal nature of reproduction, seasonal decline in semen quality, improper estrus expression including silent heat, long ovulation time, and prolonged post-partum estrus. Cruz (1998) felt that low efficiency of AI in water buffaloes is more related to human factors and hence the efficiency in AI can be improved by proper detection of the estrus, proper semen handling, the appropriate management and nutrition after the insemination of buffaloes, and post partum. Likewise, Vale (1997) was of the view that difficulty in buffalo AI is the application of the method in the field and not the semen technology. With the recent techniques of estrus detection by measuring vaginal electrical resistance (Markandeya et al., 1993; Gupta, 1998; Gupta and Purohit, 2001), milk and plasma progesterone assay (Abbas et al., 1981), laparoscopy (Jainudeen et al., 1983), ultrasonography (Manik et al., 1992) etc. and better management protocols, the reproductive efficiency is likely to improve in the near future. Global priorities for trait propagation in the river

buffaloes as well as swamp buffaloes need to be fixed up and cryopreserved semen be used on a larger scale. Crossbreeding between the swamp buffalo and river breeds are often aimed at producing milk in addition to meat and draught in the crossbreds. as reported in China and the Phillippines (Xiao Yongzuo. 1989; Mamongan et al.. 1994).

The conception rate with natural service. chilled semen and frozen semen have been reported to be more than 60. 35-60 and 25-45%. respectively (Jainudeen and Hafez. 1993c; Agarwal and Shankar. 1994). The conception rate in Surti buffaloes. after insemination with semen frozen in different dilutors varied between 37.5% to 59.1% (Dhami and Kodagali. 1990; Dhami et al.. 1994). Different cooling procedures (Dhami and Sahni. 1994) or thawing procedures (El-Amrawi. 1997) can improve conception rates up to 65%. Villa and Fabbri (1993) suggested by their trials on Italian buffaloes using semen frozen in a commercial dilutor Laiciphos® (IMV) that the high variation in the conception rates (30.5 to 57.1%) is because of the strong influence of environmental factors. The low conception rates to AI in buffaloes could also be due to the small size of uterine body compared to cows. leading to inadvertant deposition of semen into one of the uterine horns (Zicarelli et al.. 1997b). According to Vale (1997). a pregnancy rate higher than 50% can be considered as good after insemination with frozen thawed buffalo bull semen. Inseminating twice during the estrus at 6-8 h is considered a good strategy to improve conception rates in buffaloes (Rao and Venkataramulu. 1994).

CONTROLLED BREEDING

Controlled breeding is mainly achieved through control of estrus. ovulation and parturition. Gordon (1997) elucidated the use of management strategies and medicaments for reproductive manipulation of buffaloes. The medication included prostaglandins and progesterone. The use of PGF_2 alpha or its analogues administered intramuscularly in two doses 11 days apart in estrus control in buffaloes (Rao. 1979; Diaz et al.. 1994) or in single dose (Cruz et al.. 1992) or in combination with GnRH for predetermined AI (Aquino et al.. 1989) have been reported. Alternatively. intravulvo-submucosal (IVSM) route has been advocated by various workers for reduction of the dose and hence the cost (Rao and Rao. 1988; Rao and Venkatramaiah. 1989; Dhaliwal and Sharma. 1990). This route is demonstrated to have a delayed but comparable response.

Intravaginal and ear implants impregnated with progesterone have been alternative means of estrus control and synchronization (Singh et al.. 1994) in the water buffalo. Such implants. coupled with other treatments have also shown promise for induction of cyclicity in buffaloes during the summer season (Lohan et al.. 2000). Similarly GnRH treatments are also known to induce emergence of a new follicular wave in the buffalo (Kohram et al.. 2000). Buffaloes in the Indian sub-continent are known to have distinct reproductive rythyms during certain seasons of the year. These are considered to be due to higher prolactin levels. Use of anti-prolactinaemic drugs like bromocryptine. have shown promise in suppression of summer anestrus in buffaloes and so have been better management protocols. Studies on melatonin profiles have shown remarkable seasonal difference in both buffalo cows and heifers (Borghese et al.. 1994). The administration of melatonin has been reported to initiate ovarian activity during post-partum anestrus in buffaloes (Hassan et al.. 2000). Further studies on this aspect are suggested. Immune regulation of ovarian function has been studied recently (Ramadan et al.. 2000) and suggests that ovulation is an inflammatory process of immune origin. The importance of management in improving reproduction in buffaloes has been recognized and continuous exposure of buffalo cows to a fertile bull with grazing management enhances ovarian activity and improves conception rates in buffaloes (Abdalla. 2003). Studies on the molecular biology of reproduction and immunology of reproduction in the buffalo are likely to be taken up in near future.

MULTIPLE OVULATION AND EMBRYO TRANSFER (MOET)

This technique of *in vivo* embryo production exploits the genetic potential of females to accelerate the multiplication of superior animals. With the first successful transfer of an *in vivo* produced embryo in the rabbit (Heap. 1891). this technique of reproduction enhancement has been used in many domestic species. Although work on MOET in the water buffalo started some three decades ago (Drost et al.. 1983; Parnpai et al.. 1985; Alexiev et al.. 1988). because of some peculiarities the results achieved so far have been modest. The first two live calves from non-surgical transfer of bubaline embryos were born in 1983 in USA (Drost et al.. 1983) and later in India (Misra et al.. 1988). For genetic gain in traits like milk production. MOET appears to be a promising technique. Gandhi (1994) have expressed that in comparison to progeny testing the increase in expected genetic gain per year from juvenile and adult MOET schemes was 63 and 70%.

The techniques of MOET have essentially been a replicate of those used in cows and involves multiple ovulation of females by administration of gonadotropins at certain stage (luteal phase) of the oestrus cycle followed by estrus induction. *in vivo* fertilization. non-surgical recovery of embryos and transfer to suitable synchronized recipients. Poor estrus expression and poor palpable characteristics of

the ovarian structures (Madan et al., 1991), lower number of primordial follicles on the buffalo ovary (Samad and Nasseri, 1979; Danell, 1987), poor fertility and seasonality of reproduction (Drost, 1991), a lower population of antral follicles at all stages of the oestrus cycle in buffalo (Kumar et al., 1997), poor endocrine status (Palta and Madan, 1984) and a high incidence of deep atresia in ovarian follicles (Ocampo et al., 1996; Palta et al., 1998) are some of the limiting factors which have resulted in moderate responses of the MOET technology in this species. Subtle differences exist between the basic anatomy of genital organs (smaller size of ovaries in buffalo) and physiology of cattle and buffalo in terms of sexual behaviour and sexual rythyms. Ovarian function appears to be central to the difference in physiology leading to poor reproduction in this species. In addition, current knowledge of basic and seasonal patterns of follicle development in the buffalo is insufficient. Efforts at understanding the process and temporal pattern of follicular dynamics in the buffalo can help improve reproduction in this species.

Superovulation

Studies on superovulation among buffaloes have been carried out both in the river (Drost et al., 1983; Madan, 1984; Karaivanov, 1986; Mehmood et al., 1989; Alexiev, 1990; Ambrose et al., 1991; Alonso et al., 1994) and swamp buffaloes (Thungtanawat et al., 1981; Nityawardana et al., 1982; Parnpai et al., 1985; Chantaraprateep et al., 1989; Jainudeen, 1989; Venitkul, 1989) and have included work in countries such as the USA, Thailand, Bulgaria, Malaysia, Pakistan, Philippines, Egypt and India. Recently some reports have come in from countries such as Brazil (Basurelli et al., 1999; Carvalho et al., 2000) and Vietnam (Uoc et al., 1992; Nguyen et al., 1997).

Most of the studies have been oriented towards developing the most appropriate treatment for induction of multiple ovulation. These trials include tests on the type, mode, dosage and time of hormone administration. Results from such trials have in general shown that the ovulatory response in the buffalo is lower and less predictable than that achieved in cattle. Globally between 1981 to 1989 the average number of embryos recovered per donor averaged 1.16 and transferable embryos per donor 0.51 (Kamonpatana, 1990). Although the number of ovulations and total viable embryos may be as high as 4.1 and 6.0, the number of transferable embryos is lower, about 2.0 per donor per flush (Misra et al., 1994; Basurelli et al., 1999; Carvalho et al., 2000). One reason for such a discrepancy is the failure of ovulated eggs to enter the oviduct (Basurelli et al., 2000).

The effect of type and dose of hormone has been a subject of interest. Studies utilizing equine gonadotropin (Parnpai et al., 1985; Karaivanov, 1986; Vlakhov et al., 1986; Alexiev et al., 1988; Haman et al., 1992) or purified FSH, either in a declining dose (Haman et al., 1992; Joshi et al., 1992) or as a single injection (Kasiraj et al., 1992) have been conducted. However, the results demonstrate marginal differences in terms of viable transferable embryo recoveries (Misra, 1993; Basurelli et al., 1999). A general consensus is that purified FSH is superior to equine chorionic gonadotropin (PMSG) as the latter is associated with a higher incidence of unovulated follicles as also observed in cattle (Purohit et al., 2000). A recent report (Singh et al., 2000) suggests that treatment with eCG in buffaloes for superovulation results in elevated plasma inhibin from fully developed follicles which continue for a long time and culminate in inhibition of FSH leading to poor ovulation in the remaining follicles.

Studies on the time of hormone administration (Karaivanov, 1986; Mehmood et al., 1989; Beg et al., 1997; Cruz, 1998) and hormone pre-treatment have improved the embryo recoveries only marginally in the water buffalo (Cruz, 1998). Commercial scale projects involving superovulation and embryo collection in riverine buffalo have been carried out in India (Misra et al., 1994). Over a five year period, the total embryo yield improved from 1.77 to 3.83 and the total viable embryo yield from 0.92 to 2.13. Recent trials in swamp buffaloes employing 250 mg of recombinant bovine somatotropin (rBST) on day 4 of a progestagen implant followed by superovulation with FSH on days 9-11 in equal divided doses have shown no effect on the total number of embryos recovered, but significantly ($p<0.05$) increased the number of transferable embryos from 0.8±0.3 to 3.0±1.0 (Songsasen et al., 1999).

The effects of repeated superovulation have shown to produce no effect on the viable embryo recovery and the future fertility of donors. High ambient temperatures (Misra et al., 1994) and the presence of a dominant follicle (Taneja et al., 1995) have been shown to have a deleterious effect on embryo recovery, and so has the summer season (Matharoo and Singh, 1994a; Matharoo and Singh, 1994b).

GnRH agonists like deslorelin can block ovulation in the buffalo subsequent to superstimulation with FSH and hence GnRH agonist-LH protocol can be used to control follicular growth and ovulation in superstimulated buffalo (Carvalho et al., 2000). Such a protocol has shown promise in improving superovulatory response and embryo recoveries in cattle (D'Occhio et al., 1999) and can form the basis of future studies, like delaying the LH surge post superovulation or suppressing the dominant follicle before superovulation to increase the embryo recoveries. A recent report (Carvalho et al., 2002) however, have shown only marginal increase in the embryo recoveries using such a protocol for the buffalo.

Embryo Harvest

Embryos are generally recovered using standard non-surgical procedures similar to those applied in cattle. However, it is recommended that the uterus be flushed on day 5 or 6 (Drost, 1991) compared to day 7 in cattle, as embryo development is more rapid in the buffalo. Early morula to hatched blastocysts are recovered in the flushings when embryos are harvested on day 6 (Misra, 1993). Embryos have also been recovered using a laparoscope (Techakumphu et al., 1995). Embryos are transferred using procedures similar to that in cattle.

Pregnancy Rates

The reported pregnancy rates are very low. During early trials, they were between 9.2 to 17.9 percent (Drost et al., 1983). In recent years, pregnancy rates have improved from 17% (Misra et al., 1994) to 26.4% (Misra et al., 1999) in India and this follows similar reports elsewhere. Amongst the factors affecting pregnancy rates, the season, side of transfer or type of estrus have shown to have no effect, whereas asynchrony between the donor and recipient, size of the corpus luteum of recipients and quality of the embryos transferred have profound effects on the pregnancy rate (Misra et al., 1999). To date, increasing the viable embryo recovery is the largest challenge before MOET becomes commercially viable. Steps to improve the pregnancy rates should include prevention of luteolysis during the first week of transfer and reduction in the incidence of embryo mortality. There is a report in the Phillipines of a riverine buffalo calf being born following transfer in a swamp buffalo (Cruz et al., 1991).

LABORATORY PRODUCTION OF EMBRYOS

Classical *in vitro* production of embryos involves *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). Many studies have been conducted on IVM and IVF and the procedures for IVC need to be improved significantly so as to obtain transferable embryos. Poor recovery of oocytes per ovary is another major constraint in IVF protocols. The first *in vitro* fertilized buffalo calf was born a decade ago in 1991 (Madan et al., 1991). Many births have taken place thereafter.

Oocyte Recovery

Competent oocytes for *in vitro* maturation, fertilization and culture (IVMFC) are usually recovered from ovaries of live or slaughtered animals. For the latter, one or a combination of methods of follicular aspiration, dissection or ovarian slicing are used. Whatever the method of oocyte recovery it is generally agreed that in most studies buffalo ovaries yield only a small number of quality oocytes (Totey et al., 1992; Madan et al., 1994a; Das et al., 1996; Kumar et al., 1997; Samad et al., 1998) varying between 0.7 (Totey et al., 1992) to 2.4 (Kumar et al., 1997). This number is quite small compared to cattle. The reasons for such a low recovery are the same as discussed in relation to superovulation previously in this paper. One alternative to improve oocyte yield could be *in vitro* culture of pre-antral follicles. Isolation of pre-antral follicles from buffalo ovaries has been reported recently (Gupta et al., 2001) and more research on this aspect is likely to be undertaken in future. The selection of oocytes for IVM usually employs the presence and appearance of a multi-layer compact cumulus mass (Chungsoongneon and Kamonpatana, 1991; Suzuki et al., 1991; Samad et al., 1998), and homogeneous cytoplasm within the oocyte (Chauhan et al., 1998a).

Oocyte recovery from live animals involves the use of transvaginal ultrasound guided oocyte recovery (TVOR) also known as ovum pick up (OPU) for *in vitro* maturation and fertilization (Boni et al., 1994; Galli et al., 1998) or a laparotomy (Techakumphu et al., 1995). Such techniques are utilized to retrieve oocytes repeatedly from high merit female animals without sacrificing them and without disturbing their natural reproductive cycle. In a previous study the average yield of oocyte complexes was 1.33 per ovary per collection, whereas when the animals were pre-treated with FSH, oocyte recovery increased to 3.0 per ovary (Boni et al., 1994). Recent reports (Boni et al., 1997; Tavares et al., 1997; Zicarelli et al., 1997a) however, describe that oocyte recovery can be increased by prior superovulation and up to 60% of the retrieved oocytes mature following IVF thereby increasing the embryo production in the buffalo. Usually a 5 MHz ultrasound probe (Boni et al., 1994, 1996; Arlotto et al., 2000b) is used for the oocyte recovery and all follicles 6 mm or above are aspirated. Since buffaloes have 2 to 3 follicular waves (Basurelli et al., 1997) and follicular waves can be synchronized with the use of GnRH (Kohram et al., 2000) such a system can efficiently be used to increase the laboratory production of embryos in the water buffalo. The day of cycle at the time of OPU, the time of OPU relative to post-partum and the presence of a dominant follicle are known to affect the oocyte recovery (Boni et al., 1996). Live births from transfer of blastocysts derived from oocytes retrieved by OPU and matured and fertilized *in vitro* have been reported (Galli et al., 1998). In a more recent study these authors recovered 4.5 oocytes per OPU session (67 sessions on 5 buffaloes) and on *in vitro* maturation and fertilization 40.1% of the oocytes cleaved, yielding a total of 48 embryos of the freezable quality (Galli et al., 2001).

***In vitro* maturation**

Laboratory maturation of the oocytes after recovery is an essential part of the *in vitro* embryo production system.

Table 1. Culture media and supplements used for *in vitro* maturation of buffalo oocytes and the nuclear maturation rates achieved in various studies

Basic medium	Hormone supplements	Serum source	Other supplements	Nuclear maturation rates (%)	Author
TCM 199	FSH	FCS	-	86	Singh et al., 1989
TCM 199	FSH, estradiol	FCS	-	84	Chungsoongneon and Kanonpatana, 1991
TCM 199	hCG, FSH, estradiol	HS	-	41	Jainudeen et al., 1993a
TCM 199	LH, FSH, estradiol	FCS	-	80	Totey et al., 1993b
Ham's F-10	LH, FSH, estradiol	FCS		80	
TCM 199	FSH, estradiol	EBS	-	72	Totey et al., 1992
Ham's F-10	LH	EBS		77	
TCM 199	-	EBS	-	80	Madan et al., 1994a
TCM 199	FSH-P	FBS	-	78	Chauhan et al., 1996
TCM 199	-	-	buFF	67	Chauhan et al., 1997b
TCM 199	FSH-P	FBS	-	74	
TCM 199	FSH-P	FBS	-	79	Chauhan et al., 1998b
TCM 199	-	FBS	IGF-II	72	
Han's F-10	FSH, LH	EBS	-	66	Bacci et al., 1991
TCM 199	FSH, hCG, estradiol	FCS	-	58	Lu and Hsu, 1991
TCM 199	-	ECS	-	80	Samad et al., 1998
	-	EBS	-	82	
TCM 199	-	EBS	-	75	Yadav et al., 1997
	-	-	buFF	79	
TCM 199	-	-	buFF	68-71	Tajik et al., 2000
TCM 199	-	-	-	81	Samad et al., 2000
Ham's F-10	-	-	-	75	
DPBS	-	-	-	59	
TCM	FSH, LH, estradiol	-	-	68	Purohit and Sharma, 2002c
TCM	-	-	EGF, IGF-1	78	
Waymouth MB media	-	-	EGF, IGF-1	83	
DMEM	-	-	EGF, IGF-1	78	Kumar et al., 2002
Ham's F-10	-	-	EGF, IGF-1	83	

FCS-fetal calf serum; HS-human serum; FBS-fetal bovine serum; EBS-estrus buffalo serum; ECS-estrus cow serum; buFF-buffalo follicular fluid.

For recovered oocytes, in order to acquire developmental competence they should undergo nuclear and cytoplasmic maturation. Nuclear maturation assessment involves the spontaneous resumption of meiosis leading to metaphase II after 24 h of *in vitro* culture of oocytes in a suitable medium (Singh and Majumdar, 1992; Yadav et al., 1997). Assessment utilizes the staining of oocytes after fixing (acetic methanol) and giemsa's (Chauhan et al., 1996; Das et al., 1997) or aceto-orcein (Totey et al., 1992; Madan et al., 1994b; Tajik et al., 2000) staining. Alternatively the degree and extent of cumulus cell expansion can be used for such an assessment (Chauhan et al., 1997a).

Cytoplasmic maturation can also be assessed by the developmental ability following IVF. Oocytes are generally matured by culture of a group of 10-15 cumulus oocyte complexes (COC's) in 50-100 µl droplets of IVM medium under sterile paraffin oil at 38.5-39.0°C in a 5% CO_2 in air atmosphere with 90-95% relative humidity in a CO_2 incubator (Totey et al., 1992; Madan et al., 1994b; Chauhan et al., 1996). Our laboratory has recently developed an alternative technique of maturing buffalo oocytes by vaginal culture in the *in vivo* bovine vagina (Purohit and Sharma, 2002a, 2002b); oocytes are filled in a semen straw which is sealed and kept in a used CIDR-B, which is then implanted in the vagina of a cow. Only 42.2% of the oocytes reached mataphase-II stage after 24 h of culture when placed in vaginal culture compared to 72.3% reaching metaphase-II in the laboratory culture in a CO_2 incubator but the ease with which this technique can be used can prove meaningful for the future.

The culture media used for maturation of buffalo follicular oocytes are known to affect their fertilization and subsequent development (Totey et al., 1992; Samad et al., 2000). Tissue culture medium 199 (TCM 199) is the most widely used, although some studies have utilized Ham's F10, Waymouth's MB media, DPBS or DMEM. The various media and supplements used in buffalo oocyte IVM and the maturation rates are presented in Table 1. Studies on the comparative efficiencies of different sera added in buffalo oocyte IVM are very few and inconclusive.

Hormones are usually added to IVM media and known to yield high maturation rates. Beneficial effects of growth

factors on buffalo oocyte IVM under serum free conditions point towards the likely presence of these factors in serum and follicular fluid. Besides above supplements, the beneficial effects of addition of a thiol compound like cysteamine to the IVM medium to improve *in vitro* embryo production efficiency in the buffalo has been explained (Gasparrini et al., 2000). The percentage of embryos that developed to the compact morula and blastocyst stage was significantly higher (22.6%) when 50 μmol^{-1} of cysteamine was added to the IVM medium (TCM 199 supplemented with hormones and sera) compared to 14.9% when no cysteamine was added. Work on the development of completely defined serum free media has progressed in cattle (Reiger et al., 1995; Lonergan et al., 1996) and culture of buffalo oocytes under such controlled conditions, and media could prove meaningful in future research.

In vitro Fertilization

Subsequent to *in vitro* maturation, oocytes are usually incubated with pre-capacitated sperm for *in vitro* fertilization. The incubation time of oocytes with spermatozoa in a fertilization medium varies between 6 h (Madan et al., 1994a; Chauhan et al., 1997a, 1999) to 24 h (Totey et al., 1992; Samad et al., 1998) and up to 48 h (Chungsoongneon and Kamonpatana, 1991). The *in vitro* matured, cumulus-intact oocytes are preferred for *in vitro* fertilization in the buffalo (Nandi et al., 1998).

Capacitation of spermatozoa is essential before incubation of oocytes and spermatozoa in a fertilization medium. For capacitating cattle spermatozoa heparin, follicular fluid or calcium ionophore-A23183 are used. However, for the buffalo such capacitating procedures have examined only heparin at a dose rate of 10 $\mu g/ml$ (Totey et al., 1992; Chauhan et al., 1997b). Spermatozoa are either pre-incubated with heparin (Kumar et al., 1994; Pal and Dhanda, 1994) for a period of 4 to 6 h (Pavasuthipaisit et al., 1992; Madan et al., 1994a, 1994b; Chauhan et al., 1997a, 1999; Nandi et al., 1998) or heparin is added directly to the fertilization medium (Totey et al., 1992, 1996). Capacitation in buffalo sperms can be assessed by chlortetracycline fluorescence assay (CTC) or *Pisum sativum* agglutinin fluorescence assay (FITC-PSA) (Kaul et al., 2000).

Prior to *in vitro* capacitation, spermatozoa with high motility are concentrated with removal of seminal plasma. Such concentration methods either employ a 1 h swim-up (Chungsoongneon and Kamonpatana, 1991; Pavasuthipaisit et al., 1992; Madan et al., 1994a; Chauhan et al., 1997a; Nandi et al., 1998) or a density gradient method (Totey et al., 1993a, 1993b, 1996; Purohit, 2001). The motility of spermatozoa is increased by pre-treatment of spermatozoa with a motility enhancing agent such as caffeine (Totey et al., 1992; Madan et al., 1994a, 1994b; Chauhan et al., 1997a; Nandi et al., 1998) or theophylline (Jainudeen et al., 1993b). A mixture of penicillamine, hypotaurine and epinephrine (PHE) is also a good alternative (Totey et al., 1992, 1993a, 1996; Purohit, 2001).

The generally recommended sperm concentration for IVF is 2 million sperms/ml (Totey et al., 1992), however, sperm concentration as low as 0.7 million sperms/ml (Chungsoongneon and Kamonpatana, 1991) and as high as 8-10 million sperms/ml (Nandi et al., 1998) have both been reported.

Semen from different buffalo males are known to have different fertilizing capacity *in vitro* (Suzuki et al., 1992; Totey et al., 1993a). The basic fertilization medium used for buffalo oocytes is Tyrode's modified medium (TALP) (Pavasuthipaisit et al., 1992; Totey et al., 1992, 1996) or Brackett and Oliphant (BO) medium, however more studies have been concentrated on BO medium (Totey et al., 1992; Madan et al., 1994a; Ocampo et al., 1996; Chauhan et al., 1997a, 1999; Nandi et al., 1998). Fertilization *in vitro* is carried out in fertilization drops of a fertilization medium covered with sterile oil and incubated in a CO_2 incubator at temperatures and humidity similar to those used for IVM. The successful fertilization is determined either by staining of oocytes with 1% orcein in 45% acetic acid to locate the male and female pronucleas or by observing cleavage of fertilized oocytes 2 days post insemination. A recent report (Ocampo et al., 2001) depicts the fertilization of swamp buffalo oocytes using river buffalo sperm.

In vitro Culture of Cleaved Embryos

Changing needs of the developing bovine embryo generally create a 8-16 cell developmental block which is a culture induced phenomenon that is irreversible but does not result in immediate embryonic death (Wright and Bondioli, 1981). Due to a general lack of understanding as to what caused this developmental block, and a few but highly variable results obtained, led to the advent of co-culture systems (Gandolfi and Moor, 1987; Eyestone and First, 1989) in cattle. Such a co-culture system with buffalo oviductal epithelial cells (BOEC) has been widely used in the buffalo (Chungsoongeneon and Kamonpatana, 1991; Totey et al., 1992; Jainudeen et al., 1993b; Madan et al., 1994b) and known to increase embryo development to the morula and blastocyst stages. However, the proportion of cleaved embryos that develop to the blastocyst stage is still around 20-35%. A recent study utilized either cumulus cell monolayer or amniotic fluid of a developing 3 day chick embryo for co-culture of 2-4 cell zygotes. The number of zygotes that reached the expanded blastocyst stage was 10% for both the culture systems (Ocampo et al., 2001). The *in vitro* culture system requires substantial improvement in the buffalo.

EMBRYO CRYOPRESERVATION

Although some researchers have successfully frozen buffalo embryos, cryopreservation of bubaline embryos has not been attempted on a large scale. Majority of the mammalian embryos are still frozen by conventional equilibrium methods involving controlled cooling rates which require the use of freezing machines (Palasz and Mapletoft, 1996). Using such procedures, cryopreservation of buffalo embryos has been reported (Misra et al., 1992; Kasiraj et al., 1993) but pregnancy rates are very low. Transfer of 4 embryos frozen in 1.0 M glycerol to 3 recipients resulted in 1 pregnancy vs. 2 pregnancies for 5 embryo frozen in 1.4 M glycerol and transferred to 5 recipients (Misra et al., 1992). When 39 embryos were frozen in 1.4 M glycerol and transferred to equal number of recipients over a 1 year period 11 pregnancies resulted and 9 live births recorded (Kasiraj et al., 1993).

Vitrification is a recently developed technique that requires no freezing equipment and involves a physical process by which a highly concentrated solution of cryoprotectant solidifies during cooling without the formation of ice crystals. Vitrificaton has been used successfully for cryopreservation of embryos at various developmental stages (Nilmann, 1991). With the reports of simple ethylene glycol/ficoll/sucrose media (Mahmoudzadeh et al., 1996) bovine embryos have now been preserved and can be directly transferred post thaw, similar to semen. Such procedures are likely to be incorporated in cryopreservation of bubaline embryos, and hence make the system more easy and effective. The results achieved in experiments carried out so far give reason to believe that it is possible to create banks for deep frozen buffalo embryos. These banks could be used successfully for conservation of genetic resources in buffaloes, introduction of new breeds and rapid dissemination of high producing buffalo genotypes (Alexiev, 1990).

Recently attempts to vitrify buffalo oocytes have been made, with post vitrification recovery rate of 88-89% and morphologically normal. These oocytes could be further used for IVM and IVF (Dhali et al., 2000a, 2000b).

EMBRYO TECHNOLOGIES IN FERAL BUFFALOES

Work on *in vitro* embryo production in the feral species like the African buffalo (*Syncerus caffer*) essentially involves immobilization using etorphine and ultrasound guided OPU (Arlotto et al., 2000a; Gerber et al., 2000a). Using a new guide system, an average recovery of 2.5 oocytes per aspiration has been reported and mean number of oocytes per aspiration in buffalo cows varies from 2.5-2.7 with a range of 0 to 9 (Gerber et al., 2000b). Studies using slaughter house ovaries however, report an average recovery of 12 COC's per ovary by slicing or mincing (Kidson et al., 2000). However, the percentage of good quality oocytes developing to morula stage was low and did not differ significantly when collected from ovaries of calves (27.3%), non-pregnant (38.9%) and pregnant buffalo cows. Only 9.7% of oocytes recovered from pregnant cows reached up to blastocyst stage and none of the oocytes from calves or non-pregnant cows developed to blastocyst stage. Studies on ovaries from slaughter house also indicate that the number of oocytes recovered per ovary are highest (9.0 ±2.6 per ovary) from adult non-pregnant or early pregnant. African buffalo as compared to juvenile or late pregnant buffalo ovaries (Arlotto et al., 2000a). Most of the work on IVM\F\C on feral buffaloes use media developed in cattle or water buffaloes. TCM 199 is the basic medium supplemented with hormones and serum source (Smith et al., 1999; Kidson et al., 2000; Gerber et al., 2000a). Such recent work is likely to play an important role in the preservation of wild species, like the bison. However, concentrated efforts in maturation of oocytes need to be developed substantially. Reports on the superovulation and embryo transfer in the feral buffaloes like the wood bison (*Bison bison athabascae*) which was listed as an endangered species in Canada, and African cape buffalo (*Syncerus caffer*) have appeared only recently (Othen et al., 1999) with little or no success. Similary work on the cryopreservation of epididymal sperms of African buffalo have also appeared recently (Lambrechts et al., 1999).

NUCLEAS TRANSFER AND EMBRYO CLONING

The embryos obtained through cloning can be transferred to recipient animals and many animals of similar genetic make up could be obtained. The basic procedure involves the utilization of micro-manipulation and cell fusion to transfer blastomeres of multicellular embryo or somatic cell into enucleated oocytes. The nucleus of blastomere is reprogrammed in such a way that a new embryo develops (Singla, 1997; Singla et al., 1997). With the advent of using nuclei of somatic cells (Wilmut et al., 1997) cloned embryos in rabbits, mice, sheep, goats, cattle and pigs have been produced, however, there are still considerable limitations. Recently, cloning has been reported in buffaloes (Singla, 1996; Appa Rao and Totey, 1999; Kitiyanant et al., 2000; Parnpai et al., 2000; Shi et al., 2000). Buffalo fetal fibroblasts proved more efficient than granulosa cells in generating cloned swamp buffalo embryos, due to a high cleavage rate, morula and blastocyst formation (Parnpai et al., 2000). With a low rate of *in vivo* and *in vitro* procedures for embryo recovery in buffaloes, the technique of embryo cloning has immense value in the production of buffalo embryos and is likely to receive considerable attention in future.

GENDER PRESELECTION

Male and female offsprings are preferred for different purposes. Therefore gender preselection is considered advantageous as it has a significant impact on the economics of livestock production. Certain sectors of buffalo industry may benefit from a high producing male progeny, the most notable being beef stud breeders, but mostly female progeny are preferred for milk production. Possible mechanisms of altering sex include facilitating or inhibiting the transport of either X or Y chromosome bearing sperm through the reproductive tract, preferential selection of sperm at fertilization, or sex-specific death of embryo after fertilization (Rorie, 1999). Recent success using sperm cells sorted into X and Y chromosome bearing population by flow cytometry (Johnson, 1992; Windsor et al., 1993; Muster et al., 1999) may be applied to buffalo. However, cytotoxic and mutagenic effects of this technique and reduction in fertility has been reported, due to the use of UV-excitable DNA specific stain (Johnson et al., 1987; McNutt and Johnson, 1996).

The sex of an embryo can also be determined using polymerase chain reaction (PCR). Two to four cells of an embryo blastomere serve as a sample for the sexing assay. A DNA sequence that is repeated on Y chromosome is used as target for male specific DNA amplification. Since PCR is able to detect even a single copy of target DNA, repetitive DNA sequences serve to enhance the sensitivity and consequently embryo viability by minimizing the number of blastomeres required for the sexing assay (Sood, 1997).

Biopsy of morula and blastocyst cells can occur without impairing the embryonic viability. Isolation of different Y-chromosome specific DNA fragments from bovine species has been extended for the buffalo. Buffalo Y-chromosome specific repetitive DNA (BuRY. 1) has been cloned and sequenced for sexing of buffalo embryos by PCR (Appa Rao and Totey, 1999). Likewise, duplex PCR based assay using 2-4 cells from an embryo has also been reported for sexing buffalo embryos (Sood et al., 2001). Sexing could be done within 4 h and sex was assigned to all 59 embryos used in the study. PCR method of sex determination is quick, highly accurate and easy to perform. However, reports on the birth of calves from pre-sexed embryos are lacking but should occur in near future.

GENOME ANALYSIS AND GENE MAPPING

Molecular genetics has tremendously increased the understanding of the structure function and organization of genes. Genome analysis through gene mapping has progressed in farm animals especially in pigs, cattle and chicken. In buffalo some initial progress has also been made (Arvindakshan et al., 1997; Barker et al., 1997; Simonsen et al., 1998). In this context, determination of DNA sequences and monitoring of these sequences in individual animals at a low cost are fundamental to the DNA-diagnosis in animal breeding. However, as most traits are of quantitative nature in farm animals, it is important to identify quantitative trait loci (QTL) at the genomic level for use in genomic selection and application of gene transfer. Marker assisted selection would be useful in avoiding risk of getting undesirable side effects. Microsatellite variation amongst river buffaloes from Sri Lanka and Malaysia and swamp buffaloes from Thailand, Malaysia, Philippines and Australia revealed significant genetic differentiation between the river and swamp buffaloes (Barker et al., 1997). The two breed types were described to have diverged some 15,000 years ago. Studies of 11 populations of the African buffalo (*Syncerus caffer*) using mtDNA sequence data and analysis of variation at 6 micro-satellite data revealed high levels of genetic variability. The expected and observed values of heterozygosities in populations were high and varied within a narrow range. The high level of genetic variation was evenly distributed among populations and significant differentiation was only observed at the continental level (Simonsen et al., 1998). Results of analysis of buffalo genomic library suggest that its genome is rich in microsatellite type sequences (Silva et al., 2002).

GENE TRANSFER

Gene transfer is the basis of producing transgenic animals. The transgenics carry recombinant DNA within their genome, introduced by intentional human intervention (Wall, 1996). The transferred genes consists of a functional part and a regulatory element, the promoter. Transgenic production involves one of a number of techniques : micro-injection into the pronucleus of fertilized oocytes. DNA-transfer via electroporation using retroviral vectors, incorporation of sperm that carry the gene construct into the ovum at fertilization (usually via intra-cytoplasmic sperm injection, ICSI), and transformation of fetal fibroblasts followed by nucleus transfer (Cibelli et al., 1998). To increase the number of transgenics born per pregnancy, a multiplex PCR analysis is performed on embryo biopsies to identify transgenics. Thus all transgenic embryos could be selected for transfer into synchronized recipients (Hendolin et al., 2000).

Reports on production of transgenic buffalo are currently not available. The only report on genetic analysis reports on parentage assessment by DNA fingerprinting of a IVF buffalo calf (Mattapallil and Ali, 1998). Gene transfer in buffalo would be a long way and would be possible once traits of economic significance are identified and the buffalo genome mapped to a greater extent. The trait of muscular hypertrophy or double muscling have been located on

bovine chromosome 2 (Charlier et al., 1995). Since buffalo has a great significance in production of meat (which is superior in protein content and contains lower cholesterol compared to cattle) identification of such traits can be of immense value in gene transfer. Some genes for superiorities of buffaloes need to be identified like ability to use low quality roughages due to a reduced nitrogen output in the urine (Moran, 1983) greater tick resistance (Chantalakhana and Skunmun, 1998) and superior quality of milk casein. It remains to be resolved whether genes of buffalo could be of significance by transfer to homologous or heterologous species, or transgenic buffalo could be of significance for producing pharmaceutical proteins.

CONCLUSION

Understanding the ovarian and follicular dynamics appears to be pivotal to improve reproduction in the buffalo. AI in the water buffaloes needs to be improved by improving estrus detection and fixing up priorities for a global trait propagation. For the feral buffaloes, however, cryopreservation procedures need to be developed. Techniques of embryo harvest both *in vivo* and *in vitro* and, embryo cryopreservation need substantial improvement, but, with some species peculiarities this does not appear true at least for embryo production. *In vivo* oocyte recovery from live animals and further IVM/F/C should be encouraged. Methods to prevent atresia of follicles and development of follicles would also help in improving the embryo recovery rates. Amongst the recent technologies such as embryo and sperm sexing, cloning, gene transfer and genome mapping, embryo bisection and nucleus transfer appears to have immense value, as this can substantially help in improving embryo numbers.

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