Headway in Reproductive Biotechniques for Genetic Improvement of Buffaloes


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Abstract
Buffaloes are one of the major contributors in refining the rural economy, particularly in South and South-East Asia countries. There are about 177.5 million buffaloes in the world and more than 96% of which are found in Asia. Though genetic improvement of this animal resource is imperative for milk and meat, it is limited by inherent biological attributes such as, long generation interval, long inter-calving period, longer age at sexual maturity, delay in puberty, distinct seasonal reproductive pattern, silent estrous, repeat breeding and eventually low reproductive rates. However, advancement in reproductive bio-techniques offers greater prospects to achieve the desired genetic improvement in buffaloes. Artificial insemination (AI) using frozen semen is well established and manipulation of ovarian function for estrus synchronization is used in large farms commonly. Super ovulation to produce embryos in vivo yielded average transferable embryos, 1.8-2.1 per collection. With advancement, embryo production using ovum pick-up (OPU) in combination with in vitro maturation (IVM) and fertilization (IVF) has been tried to a limited extent, but it is impending to be an alternative to earlier one. Recently advanced “Hand guided Cloning Technique”, a landmark technique developed at National Dairy Research Institute (NDRI), Karnal could go a long way for faster multiplication of superior milch buffaloes in India.

Keywords: Buffalo; Reproductive biotechniques; Super ovulation; Genetic improvement.

Introduction
India is primarily an agrarian country with livestock playing a pivotal role in uplifting the socioeconomic conditions of rural masse. Presently, there are about 177.5 million water buffaloes in the world and 96% of them are found in Asia alone. India ranks first in respect of buffalo (Bubalus bubalis) population in the world and has more than 50% of the world’s buffalo population (~98 million) which contributes 56% of total milk production, 104.8 million tonnes (1). Buffaloes are multi-purpose animals and preferred over other animals due to their ability fulfill the protein requirements of human population through milk and meat, and also for their great share in providing the traction power for various agricultural purposes.
Improving the genetic potential of buffaloes for milk and/or meat has been a major concern for decades in many buffalo-producing countries, and has become a recent development focus in several more. But there are many biological conditions inherent in the buffalos such as high age at first calving, longer dry period, poor reproductive performance resulting in high service period and longer calving interval, embryonic mortality, inadequate health care, genetic slippage of superior germplasm from the breeding tracts and poor response to artificial breeding are impeding the efforts put forth in genetic improvement of buffaloes (2). However, the fast pace of developments transpiring in reproductive biotechniques can be exploited for improvement in buffaloes so as to vanquish the above cited limitations. Emerging biotechniques can be effectively employed to enhance the production performance and reproductive efficiency of buffaloes by reducing the generation interval, which in turn accelerate the genetic improvement in desired direction.

Associated to the enhancement in reproductive efficiency, techniques used to achieve genetic improvement make possible to obtain herds with better productive characteristics, such as growth rate, carcass quality, milk yield, feed conversion and precocity. Thus, multiplication of superior animals by using reproductive biotechniques can provide greater economic return. Moreover, increased reproductive rates associated to genetic improvement must be the main objective to improve buffalo productivity and farm income. However, most of them are not as efficient as in cattle. Genetic improvement is possible when potential animals are selected based on their pedigree, karyotyping (free from chromosomal aberrations), tests for various diseases like; tuberculosis, brucellosis, John’s disease and any other genetic disorders. The chromosomal aberrations are one of the reasons for reduced fertility (3). In this review, hence, the focus will be given on research advances transpired in main reproductive biotechniques applied for buffalo reproduction such as AI, estrus synchronization, super ovulation, in vivo and in vitro production (IVP) of embryos, embryo transfer, cloning, semen and embryo sexing. Along with the advancement made, the prospective areas of research one should be aimed to make these advanced reproductive biotechniques, of buffalo in particular and livestock’s as a whole, as more efficient and economical for their wider application will be deliberated.

Artificial Insemination (AI) and Associated Techniques: The quality of semen before and after freezing should be evaluated by various parameters before a bull is introduced for AI programme (4, 5, 6). AI in either natural or synchronized estrus is the earliest reproductive biotechnique in farm animals including buffalo. For many reasons, AI can be used in buffalo, to inseminate magnitudes of female using proven bull, to use a male genetic material separated by distance or time, to overcome difficulties of physical breeding, to control the offspring’s paternity, to synchronize births, to avoid injuries of natural mating, and to avert the need to rear a male (such as for small numbers of females or in species whose fertile males may be difficult to manage).

Yet, the technique of AI in overall genetic improvement programs of water buffalo is not exploited widely. The reported low efficiency of AI in water buffaloes is mainly the result of human factors such as inability to detect estrus properly, improper handling and usage of semen in the field by technicians, and most common of all, poor management and nutrition of inseminated animals. Therefore, the development of a sustainable and effective delivery system of
AI service is the only panacea to the wide-scale use of this technique, particularly in relatively dispersed buffalo populations in most of the small farms in Asia. Many researchers have underpinned various techniques to produce quality semen and thereby the better pregnancy rates following AI in water buffaloes. Some of the alternate techniques of AI to accomplish higher pregnancy rate are deliberated below.

Fixed-time artificial insemination (FTAI): The AI in bovine has been widely pursued and successfully used in breeding programs around the world. However, FTAI has been used to limited extent in buffaloes due to certain difficulties in the estrus detection and imperfect timing of AI. The failure to detect estrus is one of the main factors that impair reproductive development in artificially inseminated herds. Therefore, the use of protocols that do not require the estrus detection contributes to wax the use of AI in buffalo herds. Hence, in this review we will consider two hormonal treatments that endorse FTAI.

Synchronization of ovulation using GnRH (Gonadotropin Releasing Hormone) and prostaglandins (PG) for FTAI: In cattle, “Ovsynch” method has demonstrated efficient synchronization of ovulation and production of satisfactory pregnancy rates in the inseminated herd, without the need of estrus detection. This protocol has also been tested in buffaloes, in sequential follicular dynamics and field studies buffaloes received a treatment based on GnRH and PG according to the protocol scheduled. In these experiments it was verified that buffaloes respond to hormonal treatment, in which a new follicular wave emerged due to the ovulation of dominant follicle present at the time of the first GnRH. On day 7, buffaloes respond to PGF2α (luteolysis), and on day 9 around 80% have a synchronized ovulation within 12 hours. Additionally, a pregnancy rate (PR) of about 50% can be obtained during the breeding season, in cycling buffaloes.

Synchronization of ovulation using progesterone and/or progestin plus estradiol: The Ovsynch protocol has been demonstrated to be efficient during the breeding season in cycling buffaloes. However, the same protocol has resulted in the low pregnancy rates of 6.9 to 28.2% during off-season breeding in anestrous buffaloes. Several studies were conducted in order to establish an appropriate protocol for the off-season breeding (7, 8). The association of progesterone/progestin to estradiol in the beginning of the protocol (day 0) has been demonstrated to be effective in inducing a new follicular wave, due to the suppression of both follicle stimulated hormone (FSH) and luteinizing hormone (LH), promoting atresia of all follicles present in the ovary. Treatment of postpartum anestrous cows with progesterone results in greater follicular fluid and circulating concentrations of estradiol, increased pulsatile release of LH and increased numbers of receptors for LH in granulosa and theca cells in pre-ovulatory follicles, compared with untreated animals (9). Further, a short period of elevated progesterone concentrations during anestrus period is important for the expression of estrus as well as subsequently normal luteal function. On day 9, the progesterone device was removed and a PGF2α and an eCG (equine chorionic gonadotropin) dose was administered to decrease the progesterone levels in circulation and to improve the follicular growth, which is compromised in non cycling buffaloes during the off-season breeding. On day 11 the GnRH promotes a synchronized ovulation. In previous studies, the eCG treatment improved ovulation and pregnancy rate in anestrous females (10). These protocols resulted in approximately 50% of pregnancy at first insemination, which compel
their use in anestrous buffaloes around the year, without detecting the estrus. This strategy of breeding the buffaloes during the off season is economically viable to suffice the market demands of milk and its products.

**In vivo Production of Buffalo Embryos:** The second advancement made in the field of reproductive biotechnology is the *in-vivo* and *in-vitro* production of embryos. In both systems, still immense scope is there to improve efficiency so that the cost of production of a calf will be reduced. Methods for in vivo embryo production are single ovulation and embryo transfer (SOET) and multiple ovulation and embryo transfer (MOET). In SOET embryos are produced *in-vivo* without any superovulatory treatments using exogenous hormones. Therefore, no disturbance in the physiological milieu of the donor animals is expected. Singla and Madan (1990) produced the embryos through SOET with 60% efficiency (11). Although, the efficiency of the flushing was limited by the animal’s estrous cycle, harvested embryos were of high quality. Depending on the efficiency of collection and success of transfers, a donor can potentially produce 3-6 calves per year. Currently, MOET is commonly used method over SOET and hence we are describing this method and known factors affecting.

MOET in combination with AI was the first advanced concept in the late 1970s, to achieve more rapid gains in cattle genetics than are provided by conventional progeny testing (12). Thereafter, superovulation through the exogenous hormones administration was used in buffaloes to produce the genetically superior embryos at higher rates. However, the process relied mostly on protocols that were originally used in cattle (11). Superovulation followed by insemination of the donor, embryo collection and their transfer have allowed researchers and dairy producers to obtain multiple offspring from only a few genetically superior donors.

Buffalo donor presented low embryo recovery rate compared to cattle. Embryo transfer, as well, has many problems such as recipient estrus detection, presence of a corpus luteum at the time of embryo transfer and synchronization donor-receptor. Poor response to superovulation and low embryo recovery is attributed to the low primordial follicle pool in buffaloes as compared to cattle and high rate of follicular atresia. As a consequence, 10% buffalo failed to respond (0-2 corpus luteum; CL) and nearly half responded poorly (0-5 CL) to superovulation treatment (13). These problems make buffalo embryo recovery rate lower than cattle. Hence, considerable attention has been focused on developing the most appropriate treatment for induction of multiple ovulations in buffaloes. After the first report of buffalo calf birth through MOET technique in the world, many works were embarked with an aim to increase embryo production. Trials have included the studies on effects of different gonadotropin hormones, forms of medicine administration and pretreatments on superovulation in buffalo. Results from such trials were not very consistent, while ovulatory responses were found to be lower than those achieved in cattle. Pregnant mare serum gonadotropin (PMSG) and FSH were usually adopted for buffalo superovulation. There have been many researches on the effects of PMSG and FSH on superovulation, and almost all the experiments demonstrated that FSH is superior over PMSG and the transferable embryos per superovulation were 2.09 and 0.56, respectively. On the other hand, the development of a new purified FSH preparation has further improved the consistency of ovulatory response in buffaloes (14). This comes in the form of 600 mg NIH-FSH-P1 {Equivalent to National Institutes of Health (U.S.A.) Reference Standard NIH-FSH-P1} administered in 10 divided and decreasing doses at an interval of 12 hours. This is followed by PGF2α administration after
72 hours of initiation of superovulatory treatment. This superovulatory regimen resulted in an average of 4.2 total embryos and 2.1 viable embryos per flushing (14).

Some studies on the application of synchronization have been done in recent years, and many schemes have been developed such as administrations of progesterone, PGF2α+PGF2α, GnRH + prostaglandin and progesterone + eCG. Jiang et al. (5) adopted controlled internal releasing device (CIDR) PGc to synchronize native buffalo cows, and resulted in a synchronous estrus rate of 85.13%, while when the single drug of PGc and CIDR was applied, the synchronization rates were 64.18% and 73.01%, respectively (15). Liang et al. (16) reported GnRH+PGF2α+GnRH is the most effective combination for buffalo estrus synchronization and it resulted a synchronous rate of 88.46% (16). Qin et al. (17) reported that the synchronous estrus rate reached 91.7% when GnRH+PG+GnRH was adopted for buffalo estrus synchronization (17). The synchronous effect is better during breeding season, and combination of progesterone during anestrus season can promote synchronous rate. The seasons seem to affect the response to superovulatory treatment, with summer having a depressive effect. On the whole, the synchronization effect in our nation remains to be improved.

In recent years, the average yield has been greatly enhanced from less than 1 viable embryo to 2.5-3.0 but most were still in the range of 1.0-2.0. But, in isolated cases over 4.0 (32) and 5.9 transferable embryos have been reported.

**In vitro Production (IVP) of Buffalo Embryos:**
IVP can mass-produce embryos of higher genetic merit and is an efficient tool. This new approach ensures the supply of ample embryos for genetic improvement of buffalo. Several factors influence the success of *in vitro* embryo production, which either increase or decrease the yield and gestational viability of embryos. Differences between donors and recipients with respect to breed, age and other physiological factors should be taken into account during IVP.

Use of animals that are well adapted to the given environment will certainly assist to overcome the effect of environmental factors such as heat on oocyte quality and embryo yield. *In vitro* culture environment is another factor that immensely influences embryo production. There are different culture systems and culture media available for production of *in vitro* fertilized bovine embryos, and embryo yield generally varies among them. The efficiency of culture systems may also vary with labs/place, making data comparisons difficult. Therefore, it may be advisable to test what culture system or culture medium will provide better results for a given lab. For IVP of embryos, oocytes were amassed mainly from the ovaries collected at abattoirs and by OPU procedure. Collection of ovaries from abattoirs is very easy, but their genealogical records are not lucid. In contrary, the transvaginal ultrasound guided OPU allows repeated collection of oocytes from live donors of high genetic value.

**In vitro Production of Buffalo Embryos from Ovaries of Slaughtered Animals:**
The yield of immature oocyte and quality vary considerably depending, more or less, on species and health, size of the ovary, number of follicles accessible and retrieval methods. For example, the mean number of oocytes collected per ovary from buffalo and subsequently used for embryo production in vitro has been reported to vary between 0.43 and 0.70 in India (18), compared to 2.4 and 3.3 in Italy (19). The common method of IVP of embryos involves in vitro maturation (IVM) of the immature oocytes, *in vitro*
fertilization (IVF) of the mature oocytes and *in vitro* culture (IVC) of the presumptive embryos until a portion forms morulae and blastocyst which can be transferred non-surgically to recipients (ET) or cryopreserved for future use.

**In vitro maturation**: The ability of aspirated immature buffalo oocytes to mature *in vitro* is influenced to a large extent by maturation media and supplements. Many studies have divulged that the benefits from the supplements are media-dependent. Buffalo oocytes are mostly cultured in groups (5–20 oocytes) for 24 h in 50–100 ul droplets of tissue culture medium such as TCM-199 (20) or Ham’s F-10 medium (7) or minimum essential medium and Waymouth medium (21) or Modified synthetic oviductal fluid (mSOF) media (Our unpublished data) under paraffin oil in a CO₂ incubator at 38.5°C (5% CO₂ and 90–95% relative humidity). Maturation medium may be supplemented with serum from different sources (22, 23, 24), human chorionic gonadotrophin (25), FSH (26), PMSG (27), LH and estradiol 17b, either alone or in combination (28, 29), buffalo follicular fluid (30) and feeder cells (granulosa cells, oviductal epithelial cells; 23). Various growth factors such as epidermal growth factor (EGF; 31), EGF plus fibroblast growth factor (32), insulin-like growth factor-I (IGF-I; 33), insulin-like growth factor-II (IGF-II; 34) and cysteamine (35) in oocyte culture media have been shown to increase cumulus expansion, nuclear maturation and post-fertilization events. The maturation rate in vitro of buffalo oocytes are assessed by methods such as staining the oocytes (metaphase II stage; 36) and degree of expansion of cumulus cell mass (30). Oocytes with full or moderate cumulus cell expansion and those with slight or no cumulus cell expansion but having the first polar body extruded in the perivitelline space may be considered as matured (28). Buffalo oocytes complete both meiotic divisions earlier than cattle oocytes in an *in vitro* system (37).

It remains a challenge to develop techniques, which would permit a swift assessment of the maturity stage of oocyte without under-estimating the actual maturation rate. More research is needed to describe the molecular and cellular events during oocyte maturation; the role of regulatory factors, gene expression and translation on completion of buffalo oocyte maturation *in vitro*.

**In vitro Fertilization (IVF)**: IVF is carried out in Tyrode’s modified medium (MTALP) or Brackett or Oliphant (BO) or mSOF medium by *in vitro* matured buffalo oocytes co-incubated with frozen-thawed *in vitro* capacitated spermatozoa. Although fresh semen gives better fertilization rates than frozen-thawed semen, the practicality of using fresh buffalo semen in IVF is negligible due to drastic changes in buffalo semen quality with season. BO medium supported higher fertilization and cleavage rates than MTALP medium (29, 38) with rates of 29.8% - 78.2% and 27.6% - 68.5%, respectively. In mSOF based IVF medium, it was reported that the fertilization and cleavage rate as 40-70% (Our unpublished data). Swim up or Percoll gradient technique are used for *in vitro* capacitation of buffalo spermatozoa from frozen-thawed semen in media containing sperm motility enhancers (22, 39, 40, 41). A marked difference in the sperm concentrations and sperm–oocyte co-incubation period has been reported in buffalo IVF with varying fertilization and cleavage rates (10, 22, 25, 38, 39, 42). The hindrance to production of IVF embryos is the occurrence of polyspermy among IVF buffalo oocytes at higher rate. For graded increase of sperm concentration from 1, 5 and 10 x 10⁶ there was a corresponding increase in the occurrence of polyspermy from 24.0%, 43.2% and 64.0%, respectively (29). Perhaps,
this high incidence of polyspermy will be modulated by improving the efficiency at the level of oocyte maturation. One critical aspect of a buffalo oocyte IVM/IVF system, that demands meticulous work, is improving the culture system for the development and formation of viable and transferable blastocysts. The methodology and molecular mechanism of capacitation remains poorly defined in buffalo. Recent advances in experimental techniques such as monoclonal antibodies and other specific probes of cell function together with methods adopted to study sperm function directly (43) may provide valuable information for further investigation of buffalo sperm capacitation at the molecular level.

In vitro culture of embryos: Culture of buffalo embryos are carried out in ligated oviduct of rabbits or complex medium (TCM-199) supplemented with serum and somatic cells (cumulus cells (38) or oviductal epithelial cells (44) or simple defined media (45) and semi-defined media (46)). In some studies simple defined media have been used for culture of buffalo embryos (19, 45) but most investigators have used complex media containing serum and somatic cells (22, 34, 39, 47). The most common defined medium for in vitro culture is synthetic oviductal fluid (SOF) or a modified form of SOF (mSOF). Our unpublished data showed that blastocyst yield up to 35% of the cleaved embryos in mSOF media. Kumar et al. (2007) compared different media and reported that mSOFaa is the most effective medium for supporting the development of buffalo zygote to the morula and blastocyst stages (48). Supplementation of the embryo culture media with IGF-1 (49), insulin (47), cysteamine (35) and a combination of BSA, EGF and insulin transferrin selenium (ITS; 46) has been found to increase the blastocyst production and decrease the number of degenerated/arrested at the 2–16-cell stage embryos. In most laboratories, blastocyst production in vitro is 15–30% of inseminated oocytes.

Culturing embryos in somatic cell monolayer facilitated their development through the 8 to 16 cell block. Although the pattern of embryonic development up to the blastocyst stage is similar in cattle and buffalo, buffalo embryos had a faster rate of development to the blastocyst stage than cattle embryos. Buffalo embryos that complete first cleavage before 30h post insemination, are more likely to develop into blastocysts and the quality and viability of the these blastocysts are superior in terms of total cell number (TCN) than those complete first cleavage after 30h post insemination. The TCN, and trophoectoderm (TE) and inner cell mass (ICM) cell numbers are higher in blastocysts developing on or before Day 7 post fertilization than in those which develop after Day 7 (49). New concepts of in vitro cattle and human embryo culture that can be applied in buffalo IVP are the use of sequential media systems. In this system, the media components and physical components could be altered during the culture. A further development of sequential media is the use of perfusion culture as the vehicle to introduce changes in media composition.

As sub-optimal culture conditions may lead to the production of embryos with developmental abnormalities and reduced viability, renewed research into the interaction of factors contributing to the development of a viable embryo and signal transduction mechanisms influencing embryo development may assist in the understanding of the relationship between the embryo and culture environment.

In vitro Production of Buffalo Embryos aspired from Live Animals: Following successful trials of ultrasound guided oocyte aspiration from live cattle, commonly known as
Ovum Pick-Up, efforts were made to reproduce the same technique in buffalo. The transvaginal ultrasound-guided OPU technique is a noninvasive and repeatable procedure for recovering large numbers of meiotically competent oocytes from antral follicles of genetically proven live animals without sacrificing the animal for future production. Along with IVP, OPU can produce an average of 2.0±0.6 transferable embryos from per buffalo per month (50). Therefore, OPU augment the reproductive efficiency of females and expedite breeding progress when compared to abattoir-derived ovaries which have very little impact on genetic improvement although these provide a cheap and abundant source of oocytes for research and propagation of desired breed of animal. The association of OPU and IVP can be promising and feasible technique in cattle. However, in buffaloes, even after pursuing several studies (19, 50, 51), blastocyst rates are still low which ranges from 9.5% to 30.0% (50, 52). So far, the low rate of oocyte recovery (27.3% to 31.3%) and poor quality of oocyte and embryo seem to be interconnected to this inefficiency (51).

Various workers have compared the in vitro development ability of in vitro fertilized oocytes derived from abattoir ovaries and OPU. Zhang et al. (53) found out that the cleavage rates in the two groups were similar (57.73 versus 54.81 %), while the blastocyst rate of in vitro fertilized oocytes derived from abattoir ovaries was significantly higher than that of OPU (28.78 vs 21.34 ) (53). Neglia et al. (54) accounted that retrieval of good quality oocytes was lower in OPU than aspirating oocytes from slaughter house-derived ovaries but of a higher blastocyst yield (29.7% versus 19.9%) (54). Also, embryos arrested at tight morula stage (11.1% versus 22.3%) were of a lower proportion in live animals than abattoir ovaries. Pang et al., (55) studied the role of seasonal effect playing on the efficiency of OPU and found that the average number of oocytes recovered per session and cleavage rate of oocytes after IVF in various season was not different with each other, while the blastocyst rate of IVF embryos in autumn-winter (27.09%) was significantly higher than those in spring (20.95%) and summer (20.45%) (55). In buffaloes, Sa Filho et al. (52) tested recombinant bovine somatotropin (rbST) on OPU-IVP aiming to improve recovery and quality of oocytes and they found that higher number of follicles aspirated (12.2 ± 0.1 vs 8.7 ± 0.04) and oocytes retrieved (5.2 ± 0.5 vs. and 4.1 ± 0.5) in rbST treated group than control group (52). However, blastocyst rate were similar in both the groups (19.7% for bST group and 26.0% for control group). Regarding pregnancy rates of IVP embryos, higher rate was observed for fresh embryos (14.3%) than vitrified embryos (8.0%), when embryo transfer was done at fixed time, in previously synchronized donors (GnRH/PGF2α/GnRH). Further studies are still needed to develop strategies that improve embryo production in buffaloes, in an attempt to facilitate the commercial use of this technology.

Transgenesis, Nuclear Transfer and Cloning:

Genetic modifications in buffalo using transgenesis, nuclear transfer and cloning have a huge potential for animal agriculture as well as biomedical science. Transgenic animals are generated by injecting desirable DNA into the pronuclei of in vitro-matured and fertilized zygotes, which are subsequently cultured in vitro to the blastocyst stage before transferring them to the recipient. Nuclear transfer involves the introduction of the nucleus from a totipotent donor cell into matured enucleated oocytes. The resulting embryo is transferred to a surrogate mother for development to term. Nuclear transfer...
using embryonic, fetal and/or somatic cells as karyoplasts resulted in the production of cloned animals.

Transgenesis will accelerate the speed of genetic improvement and establish mammary gland bioreactors in buffalo, a potential way to improve the reproducibility and milk yield of buffalo. However, there are few reports on buffalo transgenesis. Verma et al. (56) reported transgenic embryos expressing green fluorescent protein (56). Huang et al. (57) produced transgenic buffalo embryos by chimera and nuclear transfer (NT) using buffalo embryonic germ (EG)-like cells expressing enhanced green fluorescent protein (EGFP) (57). Scientists of Animal Science and Technology of Guangxi University, China produced the world’s first transgenic cloned buffalo male twin calves on 19th December, 2010 in Guangxi by lentivirus-mediated gene transfer. Somatic cell nuclear transfer (SCNT) is an emerging technology with many applications in animal breeding, from multiplying superior genotypes to making genetically engineered animals and genotyping to select the best genomes for breeding. The researches in buffalo SCNT has been started and got some successful outcome. Shi et al. (58) transferred 42 SCNT embryos into 21 recipients and got the first live cloned buffalo in the world (58). Yang et al. (59) reported the research on swamp and river buffalo inter subspecies SCNT, the SCNT embryos were cryopreserved and then transferred into 15 recipients, 5 recipients were confirmed to be pregnant, and one recipient delivered a cloned river buffalo (59).

Conventional Cloning Technique that was employed for the production of cloned sheep “Dolly” demands sophisticated and expensive equipments like micromanipulators etc. Hence, the scientists of Animal Biotechnology Centre (ABTC) at NDRI, Karnal have developed the landmark technique called “Hand guided Cloning Technique” which is an advanced modification of “Conventional Cloning Technique” (60). This new cloning technique is a simple and less demanding in terms of equipment, time and skill. Using this technique, scientists have produced the world’s first cloned buffalo calf (60, 61), which died later, and the first live cloned buffalo is named as GARIMA. In this technique, immature oocytes isolated from ovaries were matured in vitro. Then they were denuded and treated with an enzyme to digest the zona pellucida. The oocytes were then treated with chemicals to push their genetic material to one side of the oocyte. This protruded side was then cut off with the help of “hand held fine blade” for removing the original genetic material of the oocyte. The enucleated oocytes were then electrofused with single cell taken from colony of embryonic stem cells or somatic cells. The resulting embryos were cultured and grown in the laboratory for seven days to develop them to the stage of blastocyst. The blastocysts were transferred to recipient buffaloes. One of the invaluable advantages of this technique is the production of sex predetermined calf, which will revolutionize the animal husbandry in near future.

Till date, four buffalo calves have produced by hand made guided technique. The first one in the world has born on 6th February, 2009, followed by second (GARIMA-I) on June 6th, 2009, third (GARIMA-II) on August 22, 2010 (61) and fourth (SHRESTH) on August 26, 2010 (61). Earlier three are female and the fourth one is male. This cloned buffalo calf is different from the earlier clone calves as, here the foster mother was provided opportunity for normal delivery with slight external assistance, the cloned calf was from ear somatic cell of 2 week old buffalo calf, and the embryo which led to successful pregnancy and normal delivery had remained frozen at -196°C for one week in liquid nitrogen.
and brought back to active life upon thawing at room temperature. The earlier three calves were born through caesarean operation and produced by using somatic, foetus and embryonic stem cell, respectively (60, 61, 62). The scientists are thinking to make the cryopreservation of embryos will need to be made as part of technique, so that the embryos could be transported and used at several places. The scientists are of the opinion that the embryonic stem cells have better cloning ability as compared to somatic cells, as such the epigenetic reprogramming of these cells is much more efficient than the somatic cells, which are already differentiated and lineage committed. Although India has a largest world’s buffalo population and they are contributing about 55% of total milk production in country, but the percentage of elite animals is very low and there is an urgent need to enhance the population of these elite buffaloes. This technology could go a long way in helping scientist to multiply the superior milch buffaloes at a faster rate in India to face the challenges of increasing demands of milk in view of the ever growing human population. Furthermore, this technology will decrease gap between supply and demand of breeding bulls in the shortest possible time.

Embryo and gamete cryopreservation:
Cryopreservation is also an important tool for the management of supernumerary embryos or embryos destined to be transferred under more appropriate conditions. This is particularly relevant in the buffalo, in which synchronization of recipients may be less efficient than in cattle, meaning that fewer surrogate females may be available for the transfer of embryos (63). Furthermore, in vitro culture and cryopreservation of cells for nuclear transfer apparently increase the chance of application of cloning technique to basic research and commercial purpose. The combination of SCNT technique with cryopreservation of cloned embryos is very important to enlarge the wellbred buffalos. However, intrinsic biological problems such as poor freezability of semen, high chilling sensitivity and high lipid content in buffalo oocytes and/or embryos have impeded the progress in cryopreservation. Few studies have been performed on embryo cryopreservation in buffalo. Cryopreservation techniques for preserving buffalo oocytes (64), spermatozoa (65), and in vivo embryos (66) have vastly improved in recent times. Recent reports have highlighted improvements in the cryopreservation of both OPU- and abattoir-derived oocytes, with morulae and blastocysts obtained following IVF of oocytes cryopreserved using slow-freezing or vitrification, although the embryo production efficiency was lower than that for fresh oocytes (67). Both conventional freezing and vitrification (68) are used for buffalo embryo cryopreservation and pregnancies from conventionally frozen (53) and vitrified (54) embryos, as well as live calves from conventionally frozen (53) and vitrified–warmed embryos (69), have been reported. Yang et al. (59) reported that 33.3% SCNT embryos survived programmed cryopreservation after thaw, and one cloned buffalo calf was obtained after transfer of thawed embryos (59). NDRI has produced the world’s first male cloned buffalo calf (SHRESTH) from cryopreserved embryo at -196°C for one week in liquid nitrogen on August 26, 2010 by landmark technique “Hand guided Cloning Technique”. Although the above researches have testified the feasibility of embryo cryopreservation in buffalo, this technique is not yet used as commercially. More works should be done in the future to promote the cryopreservation efficiency of buffalo gametes and embryos.

Sperm and embryo sexing:
Pre-selection of sex of offspring at the time of conception is the most
sought-after reproduction techniques of all time. Application of sexed semen allows the production of replacement dairy heifers from genetically superior dams. Because dairy farmers have less use for male calves, the use of sexed semen to produce only females would make buffalo production more attractive. Alternatively, because male calves exhibit higher growth rates, sexed semen can also be used for the preferential production of males for meat or draft animals. Sperm-sexing methods are based on the differential amount of DNA being present in the X- and Y-chromosome bearing sperm. Sort analysis of DNA content by flow cytometry, fluorescence in situ hybridization (FISH) and use of polymerase chain reaction (PCR) on individual sperm are effective for assessing samples to find the proportion of X and Y sperm. At the present time the flow cytometric sorting method and modified high-speed sorting provide the only fully validated means of sperm sexing in cattle. Though much of the work has been done in cattle, the information on sperm sexing in buffalo is very scanty.

Di et al. (70) cross-hybridized river buffalo spermatozoa using double color FISH with bovine X and Y-chromosome painting probes, prepared by degenerate oligonucleotide primed PCR (DOP-PCR) of laser-micro-dissected-captulated chromosomes, to investigate the possibility of using bovine probes for sexing sperm (70). Presicce et al. (71) were the first to report flow cytometric sorting and freezing of buffalo spermatozoa, as well as calves born after insemination with sexed spermatozoa in Mediterranean Italian buffaloes (71). In other studies, Murrah and Nili-Ravi buffaloes were selected for flow cytometric sperm separation, with sorting accuracies for X- and Y-bearing spermatozoa of 94% and 89%, respectively, being reported (72). Transfer of the presumed X-embryos derived from IVF using sexed spermatozoa resulted in the birth of female twins, indicating the feasibility of sperm sorting by flow cytometry, the in vitro production of sex-preselected embryos and the birth of subsequent offspring, as well as the potential use of the technology in buffaloes (72). Liang et al. (16) reported that OPU and abattoir-derived oocytes fertilized in vitro with sexed spermatozoa had similar developmental competence with regard to cleavage (57.6% v. 50.4%, respectively) and blastocyst development (16.0% v. 23.9%, respectively) rates (73). Lu et al. (72) evaluate the efficiency of AI by using the sexed sperm to produce sex-preselected calves in buffalo species (74).

Pre-implantation embryos can be sexed or screened for genetic abnormalities using techniques such as polymerase chain reaction (PCR), fluorescence in situ hybridisation (FISH) and karyotyping. Pre-implantation genetic diagnosis (PGD) allows the continued use of valuable germplasm, even from carrier animals, by screening embryos before transfer and excluding affected embryos. The sexing of buffalo embryos has been reported by various workers using the polymerase chain reaction to amplify buffalo Y-chromosome-specific primers (75, 76). Fu et al. (77) reported embryo sexing by multiplex-nested PCR and this method is extremely reliable and useful in sexing embryos before transfer (77). For example, loop-mediated isothermal amplification (LAMP), a novel DNA amplification method that amplifies a target sequence specifically under isothermal conditions, has been developed for the rapid sexing of buffalo embryos through the identification of Y-chromosome-specific sequences (78). Although embryo sexing is routinely used in the multiple ovulation embryo transfer scheme in cattle, the development of accurate embryo splitting and sexing procedures

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in buffalo requires optimization.

Results of above studies indicate the feasibility of the application of the sperm and embryo sexing technology to accelerate the genetic improvement in buffaloes. These some studies provide proof of concept for further research and wider field application in buffalo.

Conclusion

Water buffalo will remain as a crucial component of the Asian economy. As a provider of milk and source of meat, the research should be focused on genetic improvement of this important animal resource. However, the genetic improvement was impeded by the inherent biological parameters like long generation interval, which can be overpowered by using recently developed and refurbished reproductive biotechniques. Among them, AI will remain a major technology for buffalo genetic improvement programmes. Hence, there is a scope to refine AI technique with respect to its efficiency and other avoidable human related factors such as appropriate timing of AI, suitable semen handling and hygiene. Less number of ovarian follicles sets another limit to the efficient use of superovulatory regimens for buffalo production programmes. There is an urgent need, therefore, to develop more efficient systems in order to harness the available potential oocytes and maximize the utility of the superior members of the population. As an alternative, embryos can be produced in vitro through the aspiration of in vivo oocytes from superior donors and fertilizing them in vitro. In vitro oocyte fertilization and their culture appear to be the feeble point and hence efficiency of IVP of embryos has to be improved considerably to transform this technology as a practical tool in buffalo improvement. There are also various ways to maximize the efficient use of already available embryos, such as splitting the embryos prior to transfer. Once these technologies are made competent, then the current buffalo genetic resources can be ameliorated at the faster rate and the animal husbandry will be made an economical endeavor. But there is a long way before it is realized.

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