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Review Article

A review-reproductive biotechnology used in buffalo production

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Abstract

The water buffalo holds tremendous potential in the livestock sector in Asian and Mediterranean countries due to their diversified advantages. Faster multiplication of superior genotypes and the conservation of endangered buffalo breeds are crying need for the buffalo scientists. Recent advances in assisted reproductive biotechnologies, including male and female assisted technologies, offer enormous opportunities to not only improve productivity, but also to use buffaloes to produce novel products for applications to human health and nutrition. In this paper, an account of various aspects related to buffalo reproduction has been discussed. This paper included an overview of the most recent developments of reproductive biotechnologies, new strategies of reproductive management for the improvement of genetic gain and the application of newly developed reproductive technologies, such as *in vitro* embryo production, embryo and sperm sexing and cloning of buffaloes.

Keywords: water buffalo, reproductive biotechnology, assisted technology, embryo production, sperm sexing.

Introduction

Domestic water buffalo (*Bubalus bubalis*) occupy an important position in animal agriculture in many countries of the world, particularly in South Asia, the Mediterranean region of Europe and South America, and is an indispensable source of employment to the marginal farmers and landless labourers. Domestic water buffaloes are now reared in 50 countries of the world. According to FAO (2002), the total number of domestic water buffaloes are 167.126 million. Asia alone possesses about 97% (162.126 million) of the total domestic water buffaloes of the world. 70% of the total domestic buffaloes are reared in south Asia; the rest 30% are reared in South East Asia (FAO, 2002). Buffaloes are the second largest source of milk supply in the world. Over 95% (73269 thousand MT) of the world buffalo's milk is produced in Asia (FAO, 2002). The fact lying behind is the quality of buffalo milk and meat. Buffalo milk has high total solids and fat in the milk but very low cholesterol. The cholesterol contents in milk of cattle and water buffalo is 3.14 mg/g and 0.65 mg/g respectively. Similarly buffalo meat has lower cholesterol content then beef. This is why water buffaloes are getting popularity in many countries of the world.

In recent years, scientist and researchers are trying to improve buffalo reproduction through innovative approaches using various reproductive technologies. They are attempting to coordinate with different technologies such as reproductive management system that have been developed based on a thorough understanding of the endocrine, cellular and molecular factors controlling ovarian and uterine functions, selective breeding using molecular tools and cloning (Moore and Thatcher, 2006). In the last two decades interest in buffalo breeding has tremendously increased worldwide, due to the critical and important role that this species plays in many climatically disadvantaged agricultural systems. A successful buffalo breeding program highly depends on the genetic improvement that can be achieved through the application of reproductive biotechnologies. As regards biotechnologies aimed to enhance genetic progress through the maternal contribution, in buffalo, due to the low and inconsistent response to multiple ovulation and embryo transfer (MOET) treatments (Zicarelli, 1997), there has been an increasing interest worldwide in the *in vitro* embryo production (IVEP) technology. Notable reports (Nandi et al., 2002; Madan and Prakash, 2006; Presicce, 2007) have presented a comprehensive account of the information on reproductive technologies used in buffaloes. Recent advances in different reproductive techniques offer numerous possibilities for the wider exploitation and dissemination of superior buffalo genotypes. We herein present an overview of salient feature achieved in advanced reproductive biotechnologies for buffaloes in two ways- i) male assisted reproductive technologies ii) female assisted reproductive technologies. In addition, we identify emerging key issues that need priority consideration and elaborate scientific input to enable improvements in the emerging advanced reproductive techniques. Wherever necessary, the relevant examples of reproductive biotechnologies used in other livestock have also been cited.

Male assisted reproductive technology

Despite the huge opportunity to serve an artificial vagina, natural mating is still responsible for the most pregnancies occurring in buffalo herds. The use of artificial insemination (AI) in water buffaloes is still marginal for several reasons such as lack of proper heat detection, lack of truly superior progeny tested bulls and until recently, unacceptable low pregnancy rates when using both natural or synchronized oestrus (Zicarelli, 1997) and the overall poor management. The use of semen technology named artificial insemination (AI) is considered as an important and powerful tool for genetic improvement of buffalo in the continent. Its use has been most widespread in commercial dairy populations, where its rapid uptake is due to a combination of genetic, technical and economic factors. On the technical front, the development of semen extenders, straws, and liquid nitrogen freezing have made AI convenient and reliable.

The frozen semen for artificial insemination (AI) in buffaloes was first used by Bhattacharya and Srivastava, (1955) in India. Later on, several reports Roy et al., (1956); Basirov, (1964); Sahni and Roy, (1972) however, did not find encouraging success rates in the absence of appropriate technology concerning diluters, percentage of glycerol, equilibrium time, freezing methods and the AI at field level. The main reason may be for unsuccessful results were the fact that the whole technology was based on the same methodology used for bovine(Abdou *et al.*, 1978).

Some reports suggested the low resistance of buffalo sperm cells as an intrinsic factor (Bhosrekar, 1981; Ibrahim et al., 1985). In contrast compared to the bovine semen, lower concentrations of citric acid, Na+, K+ and Mg++, higher amounts of Ca++ and bicarbonate, and similar levels of fructose, inorganic phosphorus (Pi) and total antioxidant substances were found in buffalo (Singh *et al.*, 1970). Prof. Dr. Bo Crabo, a FAO-Consultant, has consolidated the feasibility of buffalo semen diluters through the program "Deep freezing preservation of water buffalo semen" with the support of the GTZ (German Agency for Technical Cooperation). This resulted in adoption of different diluters for use in deep freezing buffalo semen all over the world (Heuer, 1980). In some countries of the world, buffaloes are considered seasonal but the male seems to be less susceptible to environmental fluctuations; though thermal stress affects the quality of the semen, apart from inappropriate semen handling and poor nutrition (Misra and Sengupta, 1965; Vale et al., 1984; Vale, 1994; Vale, 1997).

After the Seminar on Buffalo Reproduction and Artificial Insemination, organized by FAO and the Swedish and the Indian Governments, in Karnal (India), 1979, some progress was achieved at different AI laboratories in the different countries , culminating with claims of superior fertility indexes upto 65 percent calving crop (Sengupta and Sukihija, 1998). Several diluters were checked carefully , mainly TRIS, TES, Skim milk, LAICIPHOS 478, milk-citrate-lactose, lactose, citrate, citric acid-serum (Roy and Ansari, 1973; Anand, 1979; Heuer, 1980; Vale et al., 1984; Vale, 1994; Vale, 1997). Fatih et al., (2010) concluded that semen of Kundhi buffalo bulls tolerate freezing and thaw stress under 15% egg yolk in tries based diluents. Thawing is an important issue that affects the semen quality for AI. Singh, (2010) conducted a research on post-thaw and semen quality. He reported, the practice of carrying frozen thawed straws insulated in cotton for insemination from AI stations to the farmers' door is likely to adversely affect the fertility in buffaloes. Brazil is the first Latin American country who took the lead in the development and practice of AI in buffaloes in early 1980s by Vale and collaborators. TRIS and TES was used by Vale et al. (1984) for successful deep freezing of buffalo semen and made the first inseminations with frozen semen, obtaining fertility indexes upon 50% based on non-return rates at 60 days post insemination. In Latin America buffaloes are raised in all countries. Although there are no official data for buffalo population for this continent, more than 5 million buffaloes of six different breeds are estimated viz. Murrah, Mediterranean, Jafarabadi, Nili-Ravi, Carabao (swamp type) and Bufalipso – the last one concentrated in Trinidad- Tobago. Yet, in Latin America the practice of AI is not so widely adopted for bovine animal including buffalo. There are several reasons such as lack of serious support by the local governments, difficulty in assessing the existing AI programs by small-holders and medium farmers and identifying constraints and formulating and assisting in the implementation of remedial measures for poor development of AI (Vale, 1994; Baruselli and Vale, 2008).

Use of sexed semen in together with *in vitro* embryo production is a potentially efficient means of obtaining offspring of preselected sex. After domestication of animals, livestock owners have desired a methodology to predetermine the sex of offspring for their herds. The ability to sort individual sperm cells into viable X- and Y-chromosome-bearing fractions made producers' sex selection dreams reality in the 1990s and now semen can be sexed with greater than 90% accuracy with use of a flow cytometric cell sorter (KeHuan *et al.*, 2013). Several concerns regarding the implementation of sexed semen technology include the apparent lower fertility of sorted sperm, the lower survival of sorted sperm after cryopreservation and the reduced number of sperm that could be separated in a specified time period. Sexing of X- and Y-chromosome bearing sperm based on the difference in DNA content is the most reliable method to produce predetermined sexed animals. Since the first report by Johnson et al., (1989) in rabbits, sexing technology has been shown to be efficacious in several species including buffalo (Johnson, 2000; Seidel and Garner, 2002; Maxwell et al., 2004; Lu et al., 2007; Liang et al., 2008).

AI with sexed sperm for production of predetermine sex offspring has been successful in cattle (Seidel Jr *et al.,* 1999); Sheep (Cran *et al.,* 1997; Hollinshead *et al.,* 2003); Horse (Buchanan et al., 2000) and Pig (Grossfeld *et al.,* 2005). Till to date, there were two reports on the application of AI with sexed sperm in buffalo, one was from (Presicce *et al.,* 2005) in which a conception rate of 42.8% was observed in Mediterranean Italian buffaloes following Ovsynch protocol and AI with sexed sperm, and another was in State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Guangxi University, Nanning, Guangxi, China laboratory (Lu *et al.,* 2010). Most recently, a successful trial of artificial insemination (AI) with sexed sperm to produce sex preselected calves in buffalo was performed in the same laboratory described as second report and this trail showed 89% of sex accuracy (Bulletin, 2013).

Female assisted reproductive technology

In the last two decades, a lot of effort has gone into the development of reproductive technologies for more efficient utilization of superior germplasm, the availability of which was very poor in buffalo species. Although the implementation of multiple ovulation and embryo transfer (MOET) programs has had very limited success in buffalo, *in vitro* production (IVP) of embryos through a combination of the techniques of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) has been successfully implemented in this species (Palta and Chauhan, 1998) and live offspring have been born after transfer of IVP embryos to suitably synchronized recipients (Chauhan *et al.,* 1997; Liang *et al.,* 2007). A successful buffalo breeding program mostly depends on the genetic improvement that can be achieved through the application of reproductive biotechnologies. The combination of Ovum pick-up (OPU) and IVEP technology is currently the most promising tool for increasing the number of transferable embryos (TE) obtainable per donor over the long term in most species(Gasparrini, 2007). In buffaloes it has been found that this technology is even more competitive in terms of embryo yields compared to MOET (Gasparrini, 2002). The low IVEP efficiency reported in buffaloes compared to cattle are due to peculiarities of the reproductive physiology of buffaloes that are not easily modifiable, such as, the low number of oocytes recruitable and their poor quality. Moreover, in the early attempts, use the IVEP system in buffalo based solely on information acquired in cattle due to the scarcity of experimental material in the majority of the countries where buffaloes are bred, with the consequent result of low IVEP efficiency (Gasparrini, 2007). The major obstacles of IVEP technology in buffaloes lie in the low number of immature oocytes that can be recovered per donor. In a controlled follicular aspiration of abattoir-collected ovaries in buffaloes allows the retrieval of good quality oocytes per ovary on average (Gasparrini *et al.,* 2000) in comparison with 10 good quality oocytes recovered in cattle (Gordon, 1994). A low number of oocytes are found in controlled experiment when OPU is performed compared to cattle (4.5 *vs* approximately 10 respectively; Galli *et al.,* 2001). Oocyte quality, that affects the IVEP efficiency in most species, plays a crucial role in buffalo, further reducing the availability of the oocytes suitable for IVEP. It is need to mention that the percentage of good quality oocytes (Grade A and B), is lower in buffalo compared to others species, not exceeding 50 % of the total oocytes recovered (Neglia *et al.,* 2003). Analysis of data collection over four years has shown an even lower proportion of good quality oocytes (33.7 % of Grade A+B), together with a higher incidence (37.9 %) of Grade C (Mishra *et al.,* 2008). Complex media, that is known to affect oocytes maturation in the most of the animal including buffalo, plays an important role in the IVEP procedure. Buffalo oocytes can be matured *in vitro* in complex media**.** The mostly used common medias are Tissue Culture Medium 199 (the most widely employed) and Ham's F-10, supplemented with sera, hormones and other additives, such as growth factors and/or follicular fluid (Gasparrini, 2002). It is known that the presence of cumulus cells in the culture medium is critical for the acquisition of developmental competence in the process of IVM, as confirmed by the significantly reduced cleavage and embryo development of denuded *vs* cumulus-enclosed oocytes following IVF (Gasparrini *et al.,* 2007). Fertilization is considered the most critical step of the IVEP procedures in buffalo, as cleavage rates lower than those obtained in other species have widely been reported (Galli *et al.,* 2001; Gasparrini *et al.,* 2003; Neglia *et al.,* 2003). Several factors that may affect the in vitro fertilization efficiency and accuracy, such as the sperm viability and capability, the adequate in vitro environment for gametes survival, the appropriate time of insemination, the duration of gametes co-incubation, the presence of cumulus cells and the acquisition of the oocyte developmental competence during the complex process of cytoplasmic maturation (Gasparrini, 2007). The success rate of embryo transfer (ET) is still lower compared to other domestic species due to the limited number of suitable recipients, that is particularly accentuated in buffalo because of the lower response to hormonal stimulation and hence to synchronization treatments(Gasparrini, 2007).Embryo cryopreservation is the best option to overcome the major problem affecting the commercial application of embryo transfer (ET) procedures. Buffalo embryos, entirely produced *in vitro*, have been successfully cryopreserved by verification, as demonstrated by their survival following *in vitro* culture (Gasparrini *et al.,* 2001) and development to term after ET (Neglia *et al.,* 2004; Sa Filho *et al.,* 2005).

Cloning

There are new dimensions in agriculture, biomedicine and animal conservation following nuclear transfer (NT), in which the nuclei of differentiated somatic cells are reprogrammed to produce cloned calves (Campbell *et al.,* 1996; Wakayama *et al.,* 1998; Wilmut *et al.,* 1999). The conventional NT cloning is a complex procedure that requires specialized laboratories, expensive equipments and reagents. To minimize the cost and to enhance the efficiency of cloning, an alternative technique called handmade cloning (HMC) has been developed. The HMC has been successfully used to clone the buffalo calves by Chinese researcher (Shi *et al.,* 2007). Since the birth of the first cloned animal (sheep: Dolly) from somatic cell nuclear transfer (Campbell *et al.,* 1996), the somatic cell nuclear transfer becomes hot fields in bio-science. In the recent year, varieties of mammals were used to somatic cell nuclear cloning, such as goat (Baguisi *et al.,* 1999), cow (Kato *et al.,* 1998), mouse (Wakayama *et al.,* 1998), pig (Polejaeva *et al.,* 2000), cat (Shin *et al.,* 2002), rabbit (Chesne *et al.,* 2002), rat (Zhou *et al.,* 2003), horse (Galli *et al.,* 2003), mule (Woods *et* *al.,* 2003), dog (Lee *et al.,* 2005) and ferret (Li *et al.,* 2006). But the somatic cell nuclear cloning of water buffalo seemed to lag behind the other species. The first somatic cell cloning of water buffalo was birthed in Guangxi University of China in 2005 (Shi *et al.,* 2007), and the first inter-subspecies cloned buffalo was birthed after embryo transfer developed from the somatic cell from swamp buffalo transferred in the de-nuclear oocyte from river buffalo in Buffalo Research Institute of Guangxi, China in 2008 (Qin *et al.,* 2010). Embryo transfer have no significant effect on increasing pregnancy rate and live calve rate, although the somatic cell cloning *in vitro* embryo production had a distinct improvement for last two decades. Lower pregnancy rate might be high abortion rate, early birth, stillborn and teratism that happen after cloned embryo transfer derived from somatic cells. Up to date, there are not many literatures available related to somatic cell nuclear cloning embryo transfer in buffalo (Qin *et al.,* 2010).

However, most recently, a bomb basting news has been published in the web site of National Dairy Research Institute (NDRI), Karnal, India. In the world, the first cloned buffaloes 'Garima' has given birth to a female calf Mahima through hand guided cloning technique. Mahima, weighing 32 kg, was born on 25th January 2013 by normal parturition. The 'Garima' was born on 22nd August 2010 as a major achievement of the NAIP funded sub-project called, "Characterisation and Differentiation of Embryonic and Spermatogonial Stem Cells in Cattle and Buffaloes". She attained early sexual maturity at 19 months of age compared to her contemporaries (around 28 months) and was inseminated with frozen-thawed semen of a progeny tested bull of NDRI on 27th March 2012, which resulted in conception (NDRI, 2013).

Conclusion

The reproductive biotechnologies have unmistakable advantages to increase the animal production, productivity of animal and animal husbandry but unfortunately disadvantageous consequence may occur if used uncontrolled leading to inbreeding and lethal malformations. Male or female assisted biotechnology including MOET, synchronization and induction of estrus and AI, as well as IVP, NT may have undesirable and sometimes serious consequences for farm animal welfare and existence. It is necessary to establish proper techniques by several laboratory and field trials. Techniques and trail result should place to discuss at international level in order to avoid the dissemination of many of these inheritance defects. The potential risk of biotechnologies for farm animal welfare and threaten for their existence should be comprehensively and systematically assessed. Biotechnological research should be multidimensional, should be logically integrated into ongoing research programmes, and should make use of appropriate and scientifically valid experimental designs and protocols. Results obtained from such research accordingly allow for developing and using the safest biotechnological methods and procedures, and, thereby, enable technological progress which is ethically justified and beneficial for society in general as well as the scientific and agricultural community. Reproductive biotechnology already discussed and it is quite feasible in buffaloes and the fertility rates have achieved more than 80 per cent of conception rates, when used throughout proper technical criteria. On this concern it seems that a wide discussion among buffalo breeders and technicians should be established to find out a way to introduce "new blood" in the form of semen or ovum.

References

Abdou, M.S.S., El • Guindi, M.M., ElMenoufy, A.A., Zaki, K., 1978. Enzymic Profile of the Semen of Bovines (*Bubalus* *bubalis* and *Bos taurus*). Zbl. Vet. Med. A 25, 222-230.

Anand, S.R., 1979. Water buffalo: dilution and preservation of semen and artificial insemination. World Review Animal Production 15, 51-55.

Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., 1999. Production of goats by somatic cell nuclear transfer. Nature biotechnology 17, 456-461.

Baruselli, P.S., Vale, W.G., 2008. Inseminación artificial em La hembra bufalina. In:Palma. G. A. (Editor) Biotecnología de la reproducción, 2nd Ed., Pugliese y Siena Publishers, p. 651-662.

Basirov, E.B., 1964. Biology of reproduction and artificial inseminaton of buffaloes, 5th Inter. Cong. Anim. Reprod. and Art. Ins. Trento, 4:10.

Bhattacharya, P., Srivastava, P.N., 1955. Studies in deep freezing of buffalo semen. 42nd Indian Science Cong., New Delhi, Vol. III.

Bhosrekar, M., 1981. Studies on buffalo semen: seasonal variation and effect of different diluents and freezing of live count and sperm abnormalities. Indian Veterinary Journal 58, 784-789.

Bulletin, B., 2013. Buffalo Bulletin, Sperm sexing in buffalo, Vol.32 (Special Issue 1): 22-30.

Campbell, K.H., Loi, P., Otaegui, P.J., Wilmut, I., 1996. Cell cycle co-ordination in embryo cloning by nuclear transfer. Reviews of reproduction 1, 40-46.

Chauhan, M.S., Katiyar, P.K., Singla, S.K., Manik, R.S., Madan, M.L., 1997. Production of buffalo calves through in vitro fertilization. Indian journal of animal sciences 67, 306-308.

Chesne, P., Adenot, P.G., Viglietta, C., Baratte, M., Boulanger, L., Renard, J.-P., 2002. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nature biotechnology 20, 366-369.

Cran, D.G., McKelvey, W.A.C., King, M.E., Dolman, D.F., McEvoy, T.G., Broadbent, P.J., Robinson, J.J., 1997. Production of lambs by low dose intrauterine insemination with flow cytometrically sorted and unsorted semen. Theriogenology 47, 267.

FAO, 2002. Production year book. Food and Agricultural Organization,Rome. 55, 213-214.

Fatih, A., Samo, M.U., Marghazani, I.B., M.A, N., Kakar;, A, N., 2010. Effect of different egg yolk levels on post thaw quality of Kundhi buffalo bull semen, Proceedings 9th World Buffalo Cgongress, Buenos Aires, 864-866.

Galli, C., Crotti, G., Notari, C., Turini, P., Duchi, R., Lazzari, G., 2001. Embryo production by ovum pick up from live donors. Theriogenology 55, 1341-1357.

Galli, C., Lagutina, I., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R., Lazzari, G., 2003. Pregnancy: a cloned horse born to its dam twin. Nature 424, 635-635.

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

Gasparrini, B., Neglia, G., Caracciolo di Brienza, V., Campanile, G., Palo, R.D., Zicarelli, L., 2001. Preliminary analysis of vitrified in vitro produced embryos. Theriogenology 55, 307-308.

Gasparrini, B., 2002. In vitro embryo production in buffalo species: state of the art. Theriogenology 57, 237-256.

Gasparrini, B., Boccia, L., De Rosa, A., Vecchio, D., Di Palo, R., Zicarelli, L., 2003. 270 In vitro fertilization of buffalo (Bubalus Bubalis) oocytes: effects of media and sperm motility inducing agents. Reproduction, Fertility and Development 16, 255-256.

Gasparrini, B., 2007. In vitro embryo production in buffalo: current situation and future perspectives. Italian Journal of Animal Science 6, 92-101.

Gasparrini, B., Attanasio, L., De Rosa, A., Monaco, E., Di Palo, R., Campanile, G., 2007. Cryopreservation of in vitro matured buffalo (*Bubalus bubalis*) oocytes by minimum volumes vitrification methods. Animal reproduction science 98, 335-342.

Gordon, I., 1994. Aspiration techniques: Old and new. Laboratory Production of Cattle Embryos. Wallingford, UK: Cab International, 71-72.

Grossfeld, R., Klinc, P., Sieg, B., Rath, D., 2005. Production of piglets with sexed semen employing a non-surgical insemination technique. Theriogenology 63, 2269-2277.

Heuer, C., 1980. Versuche zur Tiefgefrierkonservierung von Wasserbueffelsperma unter Anwendung des Filtertestes zur Samenbeurteilung. Tieraerztlichen Hochshule Hannover, PhD Dissertation.

Hollinshead, F.K., O'Brien, J.K., Maxwell, W.M.C., Evans, G., 2003. Production of lambs of predetermined sex after the insemination of ewes with low numbers of frozen “thawed sorted X-or Y-chromosome-bearing spermatozoa. Reproduction, Fertility and Development 14, 503-508.

Ibrahim, S.S., El-Azab, A.I., Racka, A.M., Soliman, F.A., 1985. The physico-chemical characteristics of the pre-ejaculate fraction, whole semen and the seminal plasma of buffalo bulls. 1st World Buffalo Cong. In, Cairo.

Johnson, L.A., Flook, J.P., Hawk, H.W., 1989. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biology of Reproduction 41, 199-203.

Johnson, L.A., 2000. Sexing mammalian sperm for production of offspring: the state-of-the-art. Animal reproduction science 60, 93-107.

Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J.-y., Doguchi, H., Yasue, H., Tsunoda, Y., 1998. Eight calves cloned from somatic cells of a single adult. Science 282, 2095-2098.

KeHuan, L., YangQing, L., Zhang, M., XiaoGan, Y., 2013. Sperm sexing in buffalo using flow cytometry. In, Buffalo Bulletin, Vol. 32, International Buffalo Information Center, pp. 22-30.

Lee, B.C., Kim, M.K., Jang, G., Oh, H.J., Yuda, F., Kim, H.J., Shamim, M.H., Kim, J.J., Kang, S.K., Schatten, G., 2005. Dogs cloned from adult somatic cells. Nature 436, 641-641.

Li, Z., Sun, X., Chen, J., Liu, X., Wisely, S.M., Zhou, Q., Renard, J.-P., Leno, G.H., Engelhardt, J.F., 2006. Cloned ferrets produced by somatic cell nuclear transfer. Developmental biology 293, 439-448.

Liang, X., Zhang, X., Yang, B., Cheng, M., Huang, F., Pang, C., Qing, G., Liao, C., Wei, S., Senatore, E.M., 2007. Pregnancy and calving rates following transfer of in-vitro-produced river and F1 (river— swamp) buffalo (*Bubalus bubalis*) embryos in recipients on natural oestrus or synchronised for ovulation. Reproduction, Fertility and Development 19, 670-676.

Liang, X.W., Lu, Y.Q., Chen, M.T., Zhang, X.F., Lu, S.S., Zhang, M., Pang, C.Y., Huang, F.X., Lu, K.H., 2008. In vitro embryo production in buffalo (*Bubalus bubalis*) using sexed sperm and oocytes from ovum pick up. Theriogenology 69, 822-826.

Lu, Y., Zhang, M., Lu, S., Xu, D., Huang, W., Meng, B., Xu, H., Lu, K., 2010. Sex-preselected buffalo (Bubalus bubalis) calves derived from artificial insemination with sexed sperm. Animal reproduction science 119, 169-171.

Lu, Y.Q., Liang, X.W., Zhang, M., Wang, W.L., Kitiyanant, Y., Lu, S.S., Meng, B., Lu, K.H., 2007. Birth of twins after in vitro fertilization with flow-cytometric sorted buffalo (*Bubalus bubalis*) sperm. Animal reproduction science 100, 192-196.

Madan, M.L., Prakash, B.S., 2006. Reproductive endocrinology and biotechnology applications among buffaloes. Society of Reproduction and Fertility supplement 64, 261-281.

Maxwell, W.M.C., Evans, G., Hollinshead, F.K., Bathgate, R., De Graaf, S.P., Eriksson, B.M., Gillan, L., Morton, K.M., O Brien, J.K., 2004. Integration of sperm sexing technology into the ART toolbox. Animal reproduction science 82, 79-95.

Mishra, V., Misra, A.K., Sharma, R., 2008. A comparative study of parthenogenic activation and in vitro fertilization of bubaline oocytes. Animal reproduction science 103, 249-259.

Misra, M.S., Sengupta, B.P., 1965. Climatic environment and reproductive behaviour of buffaloes. III. Observations on semen quality of buffalo maintained under two different housing conditions. Indian J. Dairy Sci 18, 130-133.

Moore, K., Thatcher, W.W., 2006. Major advances associated with reproduction in dairy cattle. Journal of dairy science 89, 1254-1266.

Nandi, S., Raghu, H.M., Ravindranatha, B.M., Chauhan, M.S., 2002. Production of buffalo (*Bubalus bubalis*) embryos in vitro: premises and promises. Reproduction in Domestic Animals 37, 65-74.

NDRI, 2013. National Dairy Research Institute, Karnal, India, http://icar.org.in/en/node/5692.

Neglia, G., Gasparrini, B., Caracciolo di Brienza, V., Di Palo, R., Campanile, G., Antonio Presicce, G., Zicarelli, L., 2003. Bovine and buffalo in vitro embryo production using oocytes derived from abattoir ovaries or collected by transvaginal follicle aspiration. Theriogenology 59, 1123-1130.

Neglia, G., Gasparrini, B., di Brienza, V.C., Di Palo, R., Zicarelli, L., 2004. First pregnancies carried to term after transfer of vitrified buffalo embryos entirely produced in vitro. Veterinary research communications 28, 233-236.

Palta, P., Chauhan, M.S., 1998. Laboratory production of buffalo (Bubalus bubalis) embryos. Reproduction, Fertility and Development 10, 379-392.

Polejaeva, I.A., Chen, S.-H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407, 86-90.

Presicce, G.A., Verberckmoes, S., Senatore, E.M., Klinc, P., Rath, D., 2005. First established pregnancies in Mediterranean Italian buffaloes (*Bubalus bubalis*) following deposition of sexed spermatozoa near the utero tubal junction. Reproduction in Domestic Animals 40, 73-75.

Presicce, G.A., 2007. Reproduction in the water buffalo. Reproduction in Domestic Animals 42, 24-32.

Qin, G., Chen, M., Yang, C., Li, R., Tan, Z., Pang, C., Huang, J., Zheng, H., Liang, X., Yang, B., 2010. Pregnancies Resulted from Transfer of Cloned Embryos developed from Somatic Cells in Buffaloes. Revista Veterinaria 21.

Roy, A., Srivastava, R.K., Pandey, M.D., 1956. Deep freezing of buffalo semen diluted and preserved in glycine-egg yolk medium. Indian J Dairy Sci 9, 61-62.

Roy, D.J., Ansari, M.R., 1973. Studies on freezability of buffalo spermatozoa using two freezing techniques. Indian Journal Animal science 43, 1031-1033.

Sa Filho, M.F., Carvalho, N.A.T., Gimenes, L.U., Torres-Junior, J.R., Ferriera, C.R., Perecin, F., Perini, A.P., Tetzner, T.A.D., Vantini, R., Soria, G.F., 2005. Birth of the first buffalo calves after transfer of vitrified embryos produced in vitro in America. In, Atti del 3 Congresso Nazionale sull Allevamento del Bufalo-1 Buffalo Symposium of Europe and the Americas, pp. 12-15.

Sahni, K.L., Roy, A., 1972. Deep freezing of buffalo semen. Indian Vet. J 40, 263-267.

Seidel, G.E., Garner, D.L., 2002. Current status of sexing mammalian spermatozoa. Reproduction 124, 733-743.

Seidel Jr, G.E., Schenk, J.L., Herickhoff, L.A., Doyle, S.P., Brink, Z., Green, R.D., Cran, D.G., 1999. Insemination of heifers with sexed sperm. Theriogenology 52, 1407-1420.

and fertility: an overview. Proceedings 2th World Buffalo Congress, New Delhi, vol. II, p.229-243.

Shi, D., Lu, F., Wei, Y., Cui, K., Yang, S., Wei, J., Liu, Q., 2007. Buffalos (Bubalus bubalis) cloned by nuclear transfer of somatic cells. Biology of Reproduction 77, 285-291.

Shin, T., Kraemer, D., Pryor, J., Liu, L., Rugila, J., Howe, L., Buck, S., Murphy, K., Lyons, L., Westhusin, M., 2002. Cell biology: a cat cloned by nuclear transplantation. Nature 415, 859-859.

Singh, L.N., Sengupta, B.P., Rawat, J.S., 1970. Studies on certain chemical constituents of buffalo semen. Indian journal of animal sciences 40, 1-8.

Singh, M., 2010. Effect of post-thaw cotton incubation on semen quality of buffalo bulls. Revista Veterinaria 21.

Vale, W.G., Ribeiro, H.F.L., Sousa, J.S., OhashI, O.M., 1984. Insemination artificial em búfalos (*Bubalus bubalis*) na região Amazônica. XXI. Congresso Brasileiro de Medicina Veterinaria, Belém, p.91.

Vale, W.G., 1994. Collection, processing and deep freezing of buffalo semen. Buffalo Journal 2, 65-72.

Sengupta, B.P., Sukihija, S.S., 1998. Current status of buffalo semen frozen technology

Vale, W.G., 1997. Sperm cryopresenvation. Third Course on Biotechnology of Reproduction in Buffaloes, Caserta, Italy. In: *Bubalus bubalis*- Journal Buffalo Science and Technique, suppl. 4, 129-140.

Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R., Yanagimachi, R., 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394, 369-374.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., Campbell, K.H.S., 1999. Viable offspring derived from fetal and adult mammalian cells. Clones and clones: facts and fantasies about human cloning 21.

Woods, G.L., White, K.L., Vanderwall, D.K., Li, G.-P., Aston, K.I., Bunch, T.D., Meerdo, L.N., Pate, B.J., 2003. A mule cloned from fetal cells by nuclear transfer. Science 301, 1063-1063.

Zhou, Q.I., Renard, J.-P., Le Friec, G.l., Brochard, V., Beaujean, N., Cherifi, Y., Fraichard, A., Cozzi, J., 2003. Generation of fertile cloned rats by regulating oocyte activation. Science 302, 1179-1179.

Zicarelli, L., 1997. Superovulatory response in buffaloes bred in Italy. Third Course on Biotechnology of Reproduction in buffaloes, Caserta, Italy. 167-188.