CURRENT TRENDS AND DEVELOPMENTS IN THE USE OF ASSISTED REPRODUCTIVE TECHNOLOGY AND ITS APPLICATION IN THE PHILIPPINE LIVESTOCK IMPROVEMENT PROGRAM-A REVIEW

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ABSTRACT

Assisted reproductive technology (ART) is a biotechnological tool used to achieve pregnancy in procedures such as artificial insemination, multiple ovulation and embryo transfer (MOET), *in vitro* maturation and fertilization (IVM/IVF), ovum pick up (OPU), cryopreservation, intracytoplasmic sperm injection (ICSI), sperm sexing, interspecies hybridization, transgenesis, and cloning. The successful use of ART and the different concerns associated with its application in humans have been extensively considered. Similarly, the application of ART in livestock production in developed countries has resulted in improved income and productivity. The Philippines, being a developing country, has to rely mainly on agriculture production to meet the increasing food demand of its 100 million population. To address food sufficiency and security concerns, the government has embraced the current biotechnological trend in food production, which includes research and development of ART to make it available in the livestock industry. The application of ART in the local livestock genetic improvement program could gradually speed up traditional and time-consuming breeding programs that will produce desired superior breeds in a shorter period with more reliable and promising outcomes. This paper reviews the current trends and developments in the use of ART and its application in the local livestock improvement program.

Keywords: buffaloes, *Bubalus bubalis,* assisted reproductive technology, livestock production, genetic improvement program

INTRODUCTION

Assisted reproductive technologies (ART) consist of different methods to achieve pregnancy by partial- or full-artificial means. In humans, ART have been medically practiced on couples with infertility problems, or even for the prevention of any communicable disease (Gout *et al*., 2011). The commonly used ART in humans include artificial insemination (Schwartz and Mayaux, 1982), *in vitro* maturation/*in vitro* fertilization (IVM/IVF) of oocytes (Forman *et al*., 2011; Gout *et al*., 2011), intracytoplasmic sperm injection (ICSI) (Gout *et al*., 2011), and embryo transfer (Betteridge, 2006; Dechaud *et al*., 2006; Rodriguez-Martinez, 2012). The cryopreservation of sperm, oocytes, zygotes, early cleavage-stage embryos, and blastocysts has become an integral part of most human IVF programs (Liebermann *et al*., 2002). Sperm sexing is a recent technology that allows the production

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of offspring with pre-determined sex. Through ART, pre-implantation genetic diagnosis to detect the transmission of heritable diseases is also made possible (Ménézo *et al*., 2000).

The successful outcomes of ART and the different concerns associated with its application in humans have been extensively investigated. These concerns include issues on biometrics (Bakos *et al*., 2011; Forman *et al*., 2011; McMahon *et al*., 2011; Dickey *et al*., 2012; Haavaldsen *et al*., 2012; Koning *et al*., 2012); possible transmission of diseases, or abnormalities in the outcome of any ART procedure (Gout *et al*., 2011; Barton *et al*., 2012; Pasqualotto *et al*., 2002; Schippert *et al*., 2012; Sueldo *et al*., 2012; Yilmaz *et al*., 2012); and possibilities of having genetic disorders on ART-offsprings (Batcheller *et al*., 2011; Feng *et al*., 2011; Iliadou *et al*., 2011; Collier *et al*., 2012). Biometrics prior to application of ART is a major concern due to the incidence of the large offspring syndrome (LOS) (Dickey *et al*., 2012; Haavaldsen *et al*., 2012; Hori *et al*., 2010; Walker *et al*., 1996) causing difficulty during delivery that risks the lives of both mother and baby.

Recent studies, however, find no concern on the success of ART in relation to biometrics (Bakos *et al*., 2011; Koning *et al*., 2012). LOS is characterized by more and irregular abortions, longer gestation length, higher birth weight, more dystocia, skewed sex ratio favorable to male calves, higher level of perinatal mortality, and congenital malformations (van Wagtendonk-de Leeuw, 2006). Although LOS may be uninfluenced by the adult liveweights, the birth weight trait appears not to be heritable (Walker, 1996) and could be avoided by modification of the culture systems in the production of embryos (van Wagtendonk-de Leeuw, 2006). Disease transmission was found controllable in the application of ART vis-à-vis employing needed interventions and washings to eliminate disease contamination (van Wagtendonk-de Leeuw, 2006; Gout *et al*., 2011). Similarly, there is an increased interest in the putative link between ART and genetic imprinting disorders (Batcheller *et al*., 2011; Feng *et al*., 2011; Iliadou *et al*., 2011).

In spite of the few risks posted by ART, its importance in addressing infertility problems in humans could not be underestimated. In fact, commercialization of ART is an increasing reality in both developed and developing countries. Similarly, application of ART in livestock production is enormous resulting in considerable improvement on income and productivity in the developed countries. Most reports on the achievements of various biotechnological tools for reproduction are in cattle, which include semen processing and handling for artificial insemination (AI), estrus/ovulation synchronization, super/multiple ovulation and embryo *in vivo* collection, IVM/IVF of oocytes and production of embryos, cloning by somatic cell nuclear transfer, and sperm-sexing technology to produce offsprings with pre-determined sex. Significant accomplishments have been reported in cattle and other ruminants in developed countries (Rodriguez-Martinez, 2012).

The Philippines, being an agriculture-based developing country, has to rely mainly on agriculture production to meet the increasing food demand of its population of 100 million. To address food sufficiency and security concerns, the government has embraced the current biotechnological trend in food production, which includes research and development of ART to make it available in the livestock industry. Most of the native breeds of animals here are of inferior genetics in terms of milk and meat production compared to their imported counterparts. The production of upgraded and purebred genetically superior animals is necessary to satisfy the local demand for milk and meat. The development and application of ART in local livestock production, particularly in genetic improvement, could gradually lead traditional and time-consuming genetic breeding programs into a new era thus producing desired upgraded superior breeds faster with more reliable and promising outcomes.

Application of ART in the Philippine livestock improvement program has been confined in cattle with emphasis in water buffalo (*Bubalus bubalis*), due to its important role in the agricultural economy. Here and in developing countries in Asia, the water buffalo provides milk, meat, and draft power. It is also used in some Mediterranean and Latin American countries as a source of milk and meat for specialized markets (Perera, 2011). With the water buffaloes renowned for difficulties in the implementation of ART with both males and females being problematic (Tatham *et al*., 2003; Hufana-Duran, 2008), there is a current need for faster multiplication of superior genotypes and conservation of endangered buffalo breeds. Recent advances in ART, including *in vitro* embryo production methodologies, offer enormous opportunities not only in improving productivity but also in using buffaloes to produce novel products for applications to human health and nutrition (Singh *et al*., 2009). With a comprehensive knowledge on the reproductive cycle of buffaloes (Perera, 2011), these biotechnological tools are now being used to boost buffalo production. As such, ART-capable research institutions are now taking on the challenge of assuring sufficient quality meat and milk products for the next generation. This paper reviews the current trends and developments in the use of ART and its application in the livestock improvement program in the Philippines.

Assisted reproductive biotechnologies

Artificial insemination (AI)

This technology is the artificial method of introducing semen into a female for the purpose of fertilization. Although this is also used as fertility treatment in humans, it is commonly used in livestock breeding, particularly in cattle, water buffaloes, and pigs. It remains the most important ART in developing countries (Willadsen, 1986; Wilmut, 1997; Blondin *et al*., 2009; Seidel, 2009; Lu *et al*., 2010; Morrell and Rodriguez-Martinez, 2010; Rodriguez-Martinez, 2012). It is commonly used in studies exploring animal reproductive behaviors and mechanisms (Ballester *et al*., 2007; Carvalho *et al*., 2007; Garcia *et al*., 2008; Oropeza *et al*., 2010; Underwood *et al*., 2010; Di Francesco *et al*., 2011). AI is usually employed for other ART currently being used (Vecchio *et al*., 2012; Lu *et al*., 2010; Rodriguez-Martinez, 2012).

In the Philippines, small-scale carabao raisers outnumber the large-scale raisers (http://www.bas.gov.ph/?ids=downloads_view&id=759), thus having widely dispersed animal raisers using a genetically improved bull as a breeding scheme is not practical. To improve buffalo breeds throughout the country at a faster rate, a mobile breeding scheme (i.e., through AI) is needed. The National Crossbreeding Program, spearheaded by the Philippine Carabao Center (PCC), aims to genetically transform the swamp buffaloes into milk- and meat-type animals by crossing and sustained backcrossing with the riverine breed through AI and natural mating.

Upgrading native carabaos and backcrossing crossbred buffalos with purebred riverine germplasm have been actively pursued through an expanded AI program in thousands of villages in the country. In 2011, PCC served 45,240 farmers owning 48,289 buffaloes, covering 7,793 barangays in 831 municipalities and cities in 74 provinces of the 15 regions of the country. This significantly increased the number of services by 80% over that of 2010 (PCC Annual Report, 2011). In 2013, a total of 71,273 AI services for 53,455 female carabaos were carried out covering 6,524 barangays in 860 municipalities and cities in 73 provinces. The services were made possible by 932 AI technicians composed of 428 village-based technicians, 433 from local government units, 4 from National Dairy Authority (NDA), and 67 from PCC (PCC Annual Report, 2013). A total of 1,336 calves on the ground were monitored in 2013 as a result of the 2012 breeding services of the active bulls. The AI activities will help improve the livestock industry in the country.

Multiple ovulation and embryo transfer (MOET)

This technology is used to produce breeds with superior quality in a short period. In multiple ovulations, female animals are hormonally manipulated to produce more than the normal quantity of eggs. After which, the donor is either artificially inseminated or mated with a bull. The resultant embryos are collected on Day 7 (Cruz *et al*., 1992; van Wagtendonk-de Leeuw, 2006), then either transferred fresh to the recipient female animal or cryopreserved for future use expecting 60 to 70% or 50 to 60% pregnancy rate, respectively (Betteridge, 2006).

Embryo transfer (ET) is a process where the collected embryo is placed into the uterus of a surrogate mother to establish pregnancy. It has been employed in animal husbandry to facilitate the production of genetically superior animals. In dairy management, repeat breeding is a big factor affecting economic success. The effectiveness of the transfer of frozen-thawed embryos in establishing pregnancy in repeat-breeding Holstein cattle showed that ET is an alternative in the treatment of repeat breeding (Dochi *et al*., 2008). To increase pregnancy rate following ET, luteolysis during the first week of transfer should be prevented to reduce incidence of embryonic mortality (Misra *et al*., 1999).

In the Philippines, PCC has reported one successful pregnancy in water buffalo through MOET (Jha *et al*., 1996). While Fresco (2001) reported MOET success in cattle, its application in water buffalo is limited by the poor response of this animal to superovulation treatment (Cruz *et al*., 1992).

***In vitro* maturation and fertilization (IVM/IVF)**

The introduction of IVF in 1978 has revolutionized the lives of millions of human couples previously unable to conceive (Forman *et al*., 2011). IVM/IVF is a process by which an egg is matured and then fertilized by sperm outside the body, specifically in a carbon dioxide incubator. IVF research studies have been launched as this technology provides an opportunity to produce embryos for genetic manipulation, ET, and basic research in developmental physiology. It can also be exploited for emerging biotechnologies such as transgenesis and cloning.

In livestock, the first IVF calf was born in 1981 from oocytes recovered surgically from stimulated cows (Brackett, 1982). Development of reliable culture systems includes using TCM 199 or Synthetic Oviductal Fluid (SOF) with growth factors (EGF, IGF) and hormone (FSH) supplements and gas atmospheres with low oxygen tension. Many attempts had been performed in 1990 up to 2000s to increase efficiency. Most systems used serum and co-culture and these were identified as part of the cause of the large offspring syndrome (LOS).

Advancement in IVM/IVF research was tremendous resulting in an increasing interest in *in vitro* embryo production (IVEP) technologies for faster propagation of superior germplasm. This has been demonstrated in cattle (Rodriguez-Martinez, 2012), water buffalo (Hufana-Duran *et al*., 2004; 2007; 2008), goat, pigs, sheep, and horses (Gupta and Nandi, 2011). Investigations on IVEP have been the subject of few reports with their usefulness for both basic research and commercial application (Katska-Ksiazkiewicz *et al*., 2004). IVF techniques overcome problems on male infertility providing means to use separated live sperm in a dead-dominated sperm population (Hufana-Duran *et al*., 2005), thus enabling bulls with semen of low post-thaw motilities to reproduce. One aspect that may still change in the future is automation and miniaturization of the IVF process, by better mimicking *in vivo* environment achieved by conventional microdrops/well systems and reducing the damage/influence of handling, e.g., outside-the-incubator environment and in light (van Wagtendonk-de Leeuw, 2006).

At present, IVF can be considered a mature technology wherein an average of 20 to 30% of OPU oocytes develop into transferable embryos at Day 7. In 2003 alone, more than half a million (584,762) bovine embryos were reported to have been transferred, with America still the center of most ET activities (45% of transfers). Europe and South America have each accounted for 20% of the transfers (Betteridge, 2006). The potential of ET in disease control (Stringfellow *et al*., 2004; Wrathall *et al*., 2004) and in improving livestock production has already been seen and proven in developed countries as a result of annual increases in ET activities (Figure 1) (Betteridge, 2006).

November 2004 (data kindly provided by the Canadian Holstein Association as cited by Betteridge (2006)).

Although IVEP efficiency has improved, embryo yield and development is still the subject of present researches to further improve efficiency and success rate. The source of oocytes

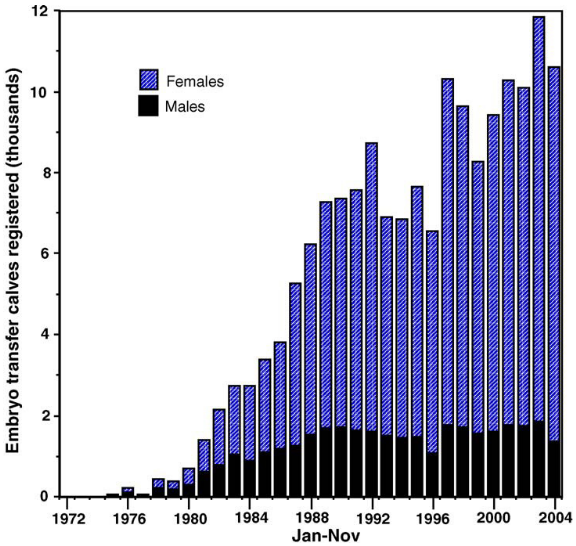

Figure 1. Embryo transfer activity in Holstein dairy cattle in Canada until November 2004 [data kindly provided by the Canadian Holstein Association as cited by Betteridge (2006)].

significantly affected post-fertilization embryo development based on the evaluation of the effects of oocyte source and quality on subsequent embryo development in water buffalo (Hufana-Duran *et al*., 2009). Also, higher overall IVEP efficiency was recorded in cattle compared with buffalo when ovaries from an abattoir were used as oocyte donors (Neglia *et al*., 2003).

In spite of the limited success of IVEP, the technology has great potential to improve the genetic progress of buffalo through the maternal lineage (Hufana-Duran *et al*., 2008). Embryo cryopreservation and the potential impact of the ovum pick-up (OPU) technique combined with IVEP critically contribute to the diffusion of ET procedures in the field and on genetic improvement of buffalos (Drost, 2007). Hence, IVEP could considerably improve the efficacy and logistics of embryo transfer and the production of genetically superior animals.

In the Philippines, initial efforts on the development and application of IVM/IVF techniques in water buffaloes were initiated at PCC and had resulted in the birth of live healthy crossbred (Ocampo *et al*., 1996) and riverine calves (Hufana-Duran *et al*., 2004; 2007; 2008) after ET. These have demonstrated the possibility of using this technology in the production of genetically superior embryos that can support the genetic improvement program. To date, IVM/IVF is the tool in the production of riverine embryos used for ET in the villages to produce riverine buffaloes, using swamp buffaloes of farmers as recipient animals (Publico, 2008). This technology provided the opportunity to use retired and slaughtered riverine buffaloes as sources of eggs and IVF in using sperm from genetically superior bulls to produce a genetically superior embryo. The establishment of a satellite embryo biotechnology laboratory of PCC in India in 2001 where river buffalo embryos were produced through IVM/IVF and transported to the Philippines in a frozen state, enabled the production of river buffalo calves in swamp buffalo recipients (Hufana-Duran *et al*., 2007). This was followed by the demonstration of twinning using ET techniques out of IVM/IVF purebred riverine buffalo embryos (Hufana-Duran *et al*., 2008).

Currently, production of embryos in the livestock industry through OPU/IVF and subsequent embryo cryopreservation has resulted in the transport of (top) genetics throughout the world thereby globalizing breeding programs. Also, transport of embryos is easier, cheaper, and allows for improved risk management of disease transmission compared to transport of live animals (van Wagtendonk-de Leeuw, 2006).

Ovum pick up (OPU)

OPU is a technique used to collect eggs from live donor-animals and have the eggs subjected to IVM/IVF to produce an embryo with desired genetics. This technique employs ultrasound-guided follicular aspiration where a vaginal probe captures the ovarian follicular images, transmits them to an ultrasound machine, and a long needle is used to aspirate the follicular oocyte. This technique allows the use of genetically superior females as sources of eggs, thus optimizing the female contribution to genetic progress and the production of genetically superior animals. Studies showed that this technique could be done two times a week to juvenile females and those that are at the early trimester of pregnancy, without compromising their reproductive performance and health (Garcia and Salaheddine, 1998; Su *et al*., 2012). In 2008, Liang *et al*. (2008) demonstrated IVEP in buffalo using sexed sperm and oocytes from OPU. Production of offspring combining OPU with other available reproductive technologies such as IVEP and vitrification for direct ET appears as a promising combination of good applicability in breeding (Rodriguez-Martinez, 2012).

To hasten the genetic improvement program in the Philippines, notwithstanding the limited number of imported riverine buffaloes that serve as breeding herd, the use of OPU could optimize the reproductive potential of the available females. Studies in Italy and China noted the potential impact of OPU technique combined with IVEP in the diffusion of ET procedures in the field and on genetic improvement of buffaloes (Gasparrini, 2002; Drost, 2007). In the current application of OPU, more efforts have to be made to optimize both repeated OPU retrieval and, particularly, the current IVM procedures, which appear to be the major limiting factors for a satisfactory IVF at present (Rodriguez-Martinez, 2012). Recently, a buffalo calf was born (Aquino *et al*., 2014) out of embryos produced from oocytes retrieved by OPU (Duran *et al.*, 2013). The sub-optimality and the costs-related issues make these techniques of little application in cattle breeding. However, their potential application in water buffalo genetic improvement cannot be underestimated particularly in swamp buffalo-dominated countries like the Philippines.

Cryopreservation

Cryopreservation is a process where cells or whole tissues are preserved by freezing them in sub-zero temperatures. Two basic techniques are currently being used in oocyte and embryo cryopreservation; the slow-freezing and vitrification. Slow-freezing is a process where extracellular water crystallizes, resulting in an osmotic gradient that draws water from the intracellular compartment till intracellular vitrification occurs. In vitrification, both intra and extracellular compartments apparently vitrify after cellular dehydration has already occurred. Owing to these differences, the terms “freezing” and “thawing” are relevant to the slow-freezing process while “cooling” and “warming” are relevant to vitrification (Saragusty and Arav, 2011).

In the area of AI, semen cryopreservation is an important technique to preserve and maximize the semen from genetically superior bulls and allow long-term storage and transport. The technique is important for the cryobanking of animal germplasm from endangered species and exploitation of genetically superior sires through AI (Hussain *et al*., 2011). Semen cryopreservation is usually done under low temperature where these can be stored for very long periods without losing their viability to fertilize. In spite of its wide application for AI, such protocol still needs further optimization to obtain higher fertilization and birth rates. There have been different studies carried out in developing and improving procedures in the collection and cryopreservation of semen, as well as the effects of extenders, freeze rates, and post-thaw quality on the fertility and success in AI (Rasul *et al*., 2000, 2001; Sansone *et al*., 2000; Sukhato *et al*., 2001; Ballester *et al*., 2007; Lessard *et al*., 2009; Hussain *et al*., 2011 Krishnakumar *et al*., 2011).

Fragmentation level (i.e., comet assay of sperm nuclear DNA) has been used for quality assessment of the cryopreserved semen samples suggesting no induced immature sperm production in dairy bulls when semen collection is performed as frequently as possible (i.e., weekly) (Mukhopadhyay *et al*., 2011). Recent work has developed a more sensitive and less costly technique of laser irradiation of spermatozoa at certain wavelengths and exposure times suitable for improvement of buffalo semen quality (Abdel-Salam *et al*., 2011). However, studies also showed that season affects semen's post-thaw viability, wherein sperm samples processed during the cooler seasons are significantly better than during the summer season (Koonjaenak *et al*., 2007). Exact temperature when the adverse effect of environment is manifested on spermatogenesis remains to be examined.

Cryopreservation also plays an important role in the preservation of oocytes and embryos. With it, the female genetics can be preserved in the form of germplasm (oocytes and embryos), ovarian tissue, or entire ovary for transplantation. In addition, germplasm can be collected and cryopreserved at different maturation stages (Saragusty and Arav, 2011). In humans, several commercial IVF laboratories have been developed in the United States, Canada, and Europe (mainly in Germany, Italy, France, and Holland) with the purpose of obtaining viable embryos from females that may not be able to produce descendants through conventional techniques (Faber *et al*., 2003). To get hold of these viable germplasm for such IVF for commercial purposes, cryopreservation is the key point in these commercial laboratories. In livestock, processing and cryopreservation of swamp buffalo's semen is one major task taken into account by PCC. At present, a total of 32 bulls are used as semen donors. In support of the expanded AI program of the country, the semen processing laboratories in PCC at CLSU/UPLB have processed a total of 105,357 doses of frozen semen. Some doses of frozen semen are also stored in PCC's Semen Bank for genetic resource conservation (PCC Annual Report, 2011).

Intracytoplasmic sperm injection (ICSI)

This is an IVF procedure where a single sperm is injected directly into the inner part of the oocyte using microinjectors or micropipettes. Among the different assisted fertilization methods used, ICSI has emerged as the ultimate technique to allow fertilization with ejaculated, epididymal, and testicular spermatozoa (Palermo, 2012). A study in humans shows that ICSI using whole sperm produces superior fertilization rates compared to ICSI using sperm heads. Although oocytes fertilized and zygote cleavage rates were not different between the two sperm sources, oocytes injected with whole sperm produced embryos of higher cell stage (Johnson *et al*., 2004). No significant difference was found on obstetric and perinatal outcomes of singleton births after ART with blastocyst transfer versus nonblastocyst transfer on women who conceived using IVF and ICSI (Fernando *et al*., 2012).

ICSI is also widely explored in livestock with most studies focusing on the recovery, improvement, and effects of oocyte quality on blastocyst development after IVF and ICSI (Kobayashi *et al*., 2006; Liang *et al*., 2008; Jacobson *et al*., 2010; Catalá *et al*., 2012). A failure of syngamy after ICSI has been confirmed molecularly through lacking expression of paternally expressed gene in swamp buffalo (Chankitisakul *et al*., 2012). ICSI has also been used in the *in vitro* development of vitrified buffalo oocytes (Liang *et al*., 2011), as well as investigated the effects of vitrification cryoprotectant treatment and cooling method on the viability and development of buffalo oocytes after ICSI (Liang *et al*., in press). In spite of the available techniques developed in the success of producing offspring using ICSI, this still warrants further research to increase success rate in producing viable and healthy offspring.

The application of ICSI in the livestock improvement program specifically in the production of pre-determined offspring is possible using sexed-sperm for fertilization. This procedure allows optimized use of sexed-sperm which is expensive and motility post-sorting is low. The rescue of genetically superior males whose sperm motility is poor can be addressed by this technique.

Sperm sexing

Several new reproductive technologies are foreseen developing further in the near future, with obvious advantages for breeding (Rodriguez-Martinez, 2012). Among these emerging ART technologies being continuously explored is the sexing of spermatozoa for directed production of desirable sex (Liang *et al*., 2008; Blondin *et al*., 2009; Seidel, 2009; Lu *et al*., 2010; Morrell and Rodriguez-Martinez, 2010; Vecchio *et al*., 2012). Sperm sexing is carried out using modified flow cytometric cell-sorting of fluorescent dye-loaded living spermatozoa. In cattle, sorting between X- and Y-chromosome-bearing spermatozoa is successful due to differences in its DNA contents (Garner and Seidel, 2008). Such technology is very promising, providing opportunities for sex selection of *in vitro*-produced embryos, surpassing the need for sex diagnosis of the embryos (which is reliably done today by DNA probing, specific for the Y chromosome, but still time-consuming and perhaps not risk-free) (Blondin *et al*., 2009; Carvalho *et al*., 2010). In spite of its importance in animal breeding, sex-sorting is too costly, slow, and yields weak spermatozoa with reduced lifespan (Lonergan, 2007; Gosálvez *et al*., 2011). With such drawback, efforts are now focused on producing competitively cheap sexed spermatozoa products for use in the livestock industry (Hayakawa *et al*., 2009; Underwood *et al*., 2010).

Most commercial sperm sorting has been with cattle where the company Cogent in the United Kingdom was the first to commercially produce sexed sperm (Garner and Seidel, 2008). Although it started slow, there has been an explosion in the production of sexed bovine sperm in 2007 and 2008, with an estimate of 4 million doses for 2008 (Sharpe and Evans, 2009). Despite its limitations such as reduced fertility, the acceptance of current sexed sperm products is increasing, specifically in the production of females for dairy farmers (Seidel, 2009).

Dairy-based enterprises in the Philippines are still in the infant stage, far behind dairy production in developed countries. With the current initiatives of the government to advance these enterprises, PCC responds to challenges not only in producing high milk-yielding breeds but also in assuring the production of breedable females, which can only be done through the use of sexed sperm during AI. While the use of sexed sperm is at the planning stage, PCC would be refining such technology to be widely available and fully disseminated to more recipients.

Interspecies hybridization, transgenesis, and cloning

ART techniques have also been used to produce hybrids, transgenic, and cloned animals. Interspecies hybridization of bovids has been successfully carried out between domestic cattle and buffalo. Such hybrids are important in improving livestock production and management of diseases that impede productivity in tropical Africa (Owiny *et al*., 2009). In spite of the feasibility of intergeneric embryo transfer between buffalo and cattle, no pregnancy was achieved after transfer of buffalo embryos to synchronized Holstein heifers. Preliminary success was achieved on nucleus transfer of swamp buffalo fetal and adult somatic nuclei into enucleated bovine oocytes and subsequent development to the blastocyst stage (Drost, 2007). A study investigating hybridization between cattle and its closest relative, the African buffalo (Syncerus caffer caffer), was carried out in an attempt to produce cattle - buffalo hybrid embryos *in vitro*. Although fertilization occurred *in vitro,* the barrier to hybridization occurs in the early stages of embryonic development (Owiny *et al*., 2009).

Transgenesis and cloning are emerging ART that have been widely applied not only in the animal industry but also in other bio-related industries. The first mammal born after nuclear transfer cloning was reported by Willadsen (1986). In spite of its enormous scientific significance, this discovery failed to trigger much public concern, possibly because the donor cells were derived from pre-implantation stage embryos. Wilmut (1997) described the successful application of almost exactly the same method, but using the nuclei of somatic cells from an adult mammal, to create Dolly, the sheep. Since 1997, a number of different species including pigs, goats, horses, cats, among others, have been cloned with the somatic cell nuclear transfer (SCNT) technique. In spite of some studies conducted on transgenesis and cloning (Uhm *et al*., 2007; Verma *et al*., 2008; Neerja *et al*., 2009; Huang *et al*., 2010; Aman *et al*., 2011; Wani, 2011), the technology has relatively low success rates. Also, there seems to be substantial problems with the welfare of some of the cloned animals, where cloning is used both within basic research and the biomedical sector. Aside from the above concerns, the ethical issues in these technologies limit their application. Nevertheless, the next step is in the development and implementation of cloning in the agricultural production system and several animals (Vajta and Gjerris, 2006). Currently, a cloned transgenic cow expressing omega-3 fatty acids, which is important to human health, has been produced (Wu *et al*., 2012).

In the Philippines, cloning by SCNT has been initiated (Atabay *et al*., 2007). Though embryos were produced in using fibroblast cells from ear skin, full-term development after embryo transfer failed. Problems associated with the failure remain an issue of investigation. Although SCNT and hybridization technologies have hinted potential application in the production/cloning of super animals that produce more milk and meat, which may further improve livestock production, problems have yet to be solved.

CONCLUSION

ART has been gradually becoming popular for humans having problems related to reproduction. Recent studies and developments in ART in humans continuously help medical personnel in the successful outcome of any ART procedures conducted. The future and benefits of such technology have also been clearly seen in the livestock industry where continuous research works are now being carried out for its smooth and efficient application, aiming for food security for the increasing human population worldwide. The use of ART has greatly revolutionized the production of superior-quality meat and milk products. The next challenge is sufficient quantity of such products to satisfy demand.

ART such as the embryo transfer technology can be very important in using genetically superior animals as donors of embryos and the native animals as surrogate mothers. Such ART could as well improve the animals' breed with respect to health and disease prevention, and prepare the animal industry in producing breeds that are adaptable to the changing climate the world is experiencing. Emerging technologies like interspecies hybridization could also improve livestock production and management, or prevent diseases. Transgenesis and cloning would enable us to develop, preserve, or even improve the desired traits that we would like to produce. A promising ART through sperm – sexing would enable us to produce the desired sex of offspring. Optimizing all these ART, the scientific community has a social responsibility to help feed the growimg population. This should be in tandem with the government, which needs to support any biotechnological option in modernizing food production as the traditional ways of farming would not be enough to satisfy the food demand. Before the commercial exploitation and use of these ART techniques in livestock breeding programs, particularly in buffalo, their initial success warrants further research up to the cellular and molecular levels to clearly and fully understand the complex mechanisms involved in animal reproduction.

Semen cryopreservation and cryobanking have recently been taken with much research focus in the application of ART in buffaloes. In the remote and hardly accessible areas of the Philippines, the AI, coupled with cryopreservation techniques, has been a widely used ART option in the livestock industry, particularly in piggery, goat, cattle, and buffalo husbandry. More work has to be done to fine-tune the protocol on every animal in order to increase efficiency, fertilization, and birth rates. Other ART such as OPU, MOET, IVEP, ICSI, Cryopreservation, and SCNT are already being performed in research laboratories in the Philippines, mostly under the experimental and trial stages. The success and efficiency of the IVEP-cryopreservation-ET have been demonstrated (Hufana-Duran *et al.*, 2004; 2007; 2008), but attempts to commercialize this technology are stalled by the inadequacy of donors of female gametes. Also, some sensitive issues are being raised by cause-oriented groups against transgenesis and cloning. While government supports the modernization of agriculture by adopting biotechnology, we may see in the future how these ART practices would boost the livestock industry in the Philippines.

REFERENCES

Abdel-Salam, Z., S.H.M. Dessouki, S.A.M., Abdel-Salam, M.A.M. Ibrahim and M.A. Harith. 2011. Green laser irradiation effects on buffalo semen. *Theriogenology*, **75**: 988-994.

Aquino F., E.P. Atabay, E.C. Atabay, M. Ocampo, P.G. Duran, P.B. Pedro, D.H. Duran, R.V. de Vera and L.C. Cruz. 2014. *In vitro* embryo production and transfer of bubaline embryos using oocytes derived from transvaginal ultrasound-guide follicular aspiration (TUFA). *Buffalo Bull.*, **32**: 545-548.

Aman, G., S. Ruchi and S. Karn. 2011. Production of cloned transgenic embryos using buffalo (*Bubalus bubalis*) embryonic stem cell-like cells isolated from *in vitro* fertilized and cloned blastocysts. *Cell. Reprogram.*, **13**: 263-272.

Bakos, H.W., R.C. Henshaw, M. Mitchell and M. Lane. 2011. Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology. *Fertil. Steril.*, **95**: 1700-1704.

Ballester, J., A. Johannisson, F. Saravia, M. Håård, H. Gustafsson, D. Bajramovic and

H. Rodriguez-Martinez. 2007. Post-thaw viability of bull AI-doses with low-sperm numbers. *Theriogenology*, **68**: 934-943.

Barton, S.E., S.A. Missmer, K.F. Berry and E.S. Ginsburg. 2012. Female cancer survivors are low responders and have reduced success compared with other patients undergoing assisted reproductive technologies. *Fertil. Steril*., **97**: 381-386.

Batcheller, A., E. Cardozo, M. Maguire, A.H. DeCherney and J.H. Segars. 2011. Are there subtle genome-wide epigenetic alterations in normal offspring conceived by assisted reproductive technologies. *Fertil. Steril*., **96**: 1306-1311.

Betteridge, K.J. 2006. Farm animal embryo technologies: achievements and perspectives. *Theriogenology*, **65**: 905-913.

Blondin, P., M. Beaulieu, V. Fournier, N. Morin, L. Crawford, P. Madan and W.A. King. 2009. Analysis of bovine sexed sperm for IVF from sorting to the embryo. *Theriogenology*, **71**: 30-38.

Carvalho, N.A.T., M. Nichi, C.E.P. Henriquez, C.A. Oliveira and P.S. Bruselli. 2007. Use of human Chorionic Gonadotropin (hCG) for fixed-time artificial insemination in buffalo (*Bubalus bubalis*). *Anim. Reprod*., **4**: 98-102.

Carvalho, J.O., R. Sartori, G.M. Machado, G.B. Mourão and M.A.N. Dode. 2010. Quality assessment of bovine cryopreserved sperm after sexing by flow cytometry and their use in *in vitro* embryo production. *Theriogenology*, **74**: 1521-1530.

Catalá, M.G., D. Izquierdo, M. Rodriguez-Prado, S. Hammami and M.T. Paramio. 2012. Effect of oocyte quality on blastocyst development after *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in a sheep model. *Fertil. Steril*., **97**: 1004-1008.

Chankitisakul, V., T. Tharasanit, N. Phutikanit, K. Tasripoo, T. Nagai and M. Techakumphu. 2012. Lacking expression of paternally-expressed gene confirms the failure of syngamy after intracytoplasmic sperm injection in swamp buffalo (*Bubalus bubalis*). *Theriogenology*, **77**: 1415-1424.

Collier, A.C., K.A. Milam, L.R.A. Rougée, A. Sugawara, Y. Yamauchi and M.A. Ward. 2012. Upregulation of *Ugt1a* genes in placentas and fetal livers in a murine model of assisted reproduction. *Placenta*, **33**: 77-80.

De Araujo Berber, R.C., E.H. Madureira and P.S. Baruselli. 2002. Comparison of two Ovsynch protocols (GnRH versus LH) for fixed timed insemination in buffalo (*Bubalus bubalis*). *Theriogenology*, **57**: 1421-1430.

Dechaud, H., T. Anahory, L. Reyftmann, V. Loup, S. Hamamah and B. Hedon. 2006. Obesity does not adversely affect results in patients who are undergoing in vitro fertilization and embryo transfer. *Eur. J. Obstet. Gyn. R. B*., **127**: 88-93.

Dickey, R.P., X. Xiong, R.E. Gee and G. Pridjian. 2012. Effect of maternal height and weight on risk of preterm birth in singleton and twin births resulting from in vitro fertilization: a retrospective cohort study using the society for assisted reproductive technology clinic outcome reporting system. *Fertil. Steril*., **97**: 349-354.

Di Francesco, S., G. Neglia, D. Vecchio, M.S. Novoa, A. Balestrieri and L. Zicarelli. 2011. Efficiency of artificial insemination in Mediterranean Italian buffalo during two seasons. *Reprod. Domes. Anim.*, **46**: 99-100.

Dochi, O., K. Takahashi, T. Hirai, H. Hayakawa, M. Tanisawa, Y. Yamamoto and H. Koyama. 2008. The use of embryo transfer to produce pregnancies in repeated-breeding dairy cattle. *Theriogenology*, **69**: 124-128.

Domingo, J.L. and J.G. Bordonaba. 2011. A literature review on the safety assessment of genetically modified plants. *Environmental International*, **37**: 734-742.

Drost, M. 2007. Advanced reproductive technology in the water buffalo. *Theriogenology*, **68**: 450-453.

Duran, P.G., D. Hufana-Duran, J.V. del Rosario, F.P. Aquino, P.B. Pedro, E.R.S. Maylem, R.V. de Vera, E.P. Atabay and L.C. Cruz. Follicular dynamics during ovum pick-up at different intervals in water buffalo, *In 10th Annual Conference of the Asian Reproductive Biotechnology Society*, Vietnam.

Faber, D.C., J.A. Molina, C.L. Ohlrichs, D.F.V. Zwaag and L.B. Ferré. 2003. Commercialization of animal biotechnology. *Theriogenology*, **59**: 125-138.

Feng, C., S. Tian, Y. Zhang, J. He, X.M. Zhu, D. Zhang, J.Z. Sheng and H.F. Huang. 2011. General imprinting status is stable in assisted reproduction-conceived offspring. *Fertil. Steril.*, **96**: 1417-1423.

Fernando, D., J.L. Halliday, S. Breheny and D. Lindsay. 2012. Outcomes of singleton births after blastocyst versus nonblastocyst transfer in assisted reproductive technology. *Fertil. Steril.*, **97**: 579-584.

Forman, E.J., N.R. Treff and R.T. Scott Jr. 2011. Fertility after age 45: From natural conception to assisted reproductive technology and beyond. *Maturitas*, **70**: 216-221.

Fresco, M.O. 2001. Scientists find twinning technology successful. *BAR Digest.*, **3**(1).

Garcia, A.R., B. Nahum, J. Lourenco, N. Costa, K. Goncalves, A. Miyazaki, M. Yoshio, C. De Andrade, A. Furugen and R.P. de Arruda. 2008. The association of medroxyprogesterone to the Ovsynch protocol for the fixed time artificial insemination of cyclic buffaloes (*Bubalus bubalis*) in the Eastern Amazon. *Acta Amazon.*, **38**: 369-377.

Garcia, A. and M. Salaheddine. 1998. Effects of repeated ultrasound-guided transvaginal follicular aspiration on bovine oocyte recovery and subsequent follicular development. *Theriogenology*, **50**: 575-585.

Garner, D.L. and G.E. Seidel Jr. 2008. History of commercializing sexed semen for cattle. *Theriogenology*, **69**: 886-895.

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

Gosálvez, J., R.A. Ramirez, C. López-Fernández, F. Crespo, K.M. Evans, M.E. Kjelland and J.F. Moreno. 2011. Sex-sorted bovines spermatozoa and DNA damage: I. Static features. *Theriogenology*, **75**: 197-205.

Gout, C., N. Rougier, P. Oger, B. Dorphin, V. Kahn, L. Jacquesson, J.P. Ayel and C. Yazbeck. 2011. Assisted reproductive technologies in HIV patients: A comprehensive review of indications, techniques and results. *Gynecol. Obstet. Fertil.*, **39**: 704-708.

Gupta, P.S.P. and S. Nandi. 2011. Isolation and culture of preantral follicles for retrieving oocytes for the embryo production: Present status in domestic animals. *Reprod. Domes. Anim.*, **47**: 513-519.

Gupta, P.S.P., S. Nandi, B.M. Ravindranatha and P.V. Sarma. 2001. Effect of commercially available PMSG on maturation, fertilization and embryo development of buffalo oocytes *in vitro*. *Reprod. Fert. Develop*., **13**: 355-360.

Gupta, P.S.P., S. Nandi, B.M. Ravindranatha and P.V. Sarma. 2002. In vitro culture of buffalo (*Bubalus bubalis*) preantral follicles. *Theriogenology*, **57**: 1839-1854.

Haavaldsen, C., T. Tanbo and A. Eskild. 2012. Placental weight in singleton pregnancies with and without assisted reproductive technology: a population study of 536 567 pregnancies. *Hum. Reprod*., **27**: 576-582.

Hayakawa, H., T. Hirai, A. Takimoto, A. Ideta and Y. Aoyagi. 2009. Superovulation and embryo transfer in Holstein cattle using sexed sperm. *Theriogenology*, **71**: 68-73.

Hori, N., M. Nagai, M. Hirayama, T. Hirai, K. Matsuda, M. Hayashi, T. Tanaka, T. Ozawa and S. Horike. 2010. Aberrant CpG methylation of the imprinting control region KvDMR1 detected in assisted reproductive technology-produced calves and pathogenesis of large offspring syndrome. *Anim. Reprod. Sci*., **122**: 303-312.

Huang, B., K. Cui, T. Li, X. Wang, F. Lu, Q. Lui, F.M. Da Silva and D. Shi. 2010. Generation of buffalo (*Bubalus bubalis*) transgenic chimeric and nuclear transfer embryos using embryonic germ-like cells expressing enhanced green fluorescent protein. *Reprod. Domes. Anim*., **45**: 103-108.

Hussain, S.A., C. Lessard and M. Anzar. 2011. Quantification of damage at different stages of cryopreservation of endangered North American bison (*Bison bison*) semen and the effects of extender and freeze rate on post-thaw sperm quality. *Anim. Reprod. Sci*., **129**: 171-179.

Hufana-Duran D., P.B. Pedro, H.V. Venturina, R.D. Hufana, A.L. Salazar Jr, P.G. Duran and L.C. Cruz. 2004. Post-warming hatching and birth of live calves following transfer of in vitro-derived vitrified water buffalo (*Bubalus bubalis*) embryos. *Theriogenology*, **61**: 1429-1439.

Hufana-Duran D., P.B. Pedro, A.L. Salazar, H.V. Venturina, P.G. Duran and L.C. Cruz. 2007. River buffalo calves (2n=50) delivered to term by swamp buffalo recipients (2n=48) out of *in vitro*-derived vitrified embryos. *Livest. Sci*., **107**: 213-219.

Hufana-Duran D., P.B. Pedro, A.L. Salazar Jr, H.V. Venturina, P.G. Duran, Y. Takahashi, Y. Kanai and L.C. Cruz. 2008. Twin calf production in water buffaloes following non-surgical transfer of *in vitro*-produced-vitrified embryos. *Philippine Journal of Science*, **137**: 99-104.

Iliadou, A.N., P.C.J. Janson and S. Cnattingius. 2011. Epigenetics and assisted reproductive technology. *J. Intern. Med*., **270**: 414-420.

Jacobson, C.C., Y.H. Choi, S.S. Hayden and K. Hinrichs. 2010. Recovery of mare oocytes on a fixed biweekly schedule, and resulting blastocyst formation after intracytoplasmic sperm injection. *Theriogenology*, **73**: 1116-1126.

Jha, S.S, H.V. Venturina, F.A. Adriano, P.G. Duran, N. Lorenzo, O.F. Smith and L.C. Cruz. 1996. Studies on embryo movement and multiple ovulation and embryo transfer (MOET) in water buffaloes, p. 48-49. *In Abstracts of Water Buffalo Researches in the Philippines 1981-1995 PCC-DA*.

Johnson, J.E., H.L. Higdon, D.W. Blackhurst and W.R. Boone. 2004. Expectation for oocyte fertilization and embryo cleavage after whole sperm versus sperm head intracytoplasmic sperm injection. *Fertil. Steril*., **82**: 1412-1417.

Katska-Ksiazkiewicz, L., B. Rynska, B. Gajda and Z. Smorag. 2004. Effect of donor stimulation, frozen semen and heparin treatment on the efficiency of in vitro embryo production in goats. *Theriogenology*, **62**: 576-586.

Kobayashi, M., E.S. Lee and Y. Fukui. 2006. Cysteamine or Beta-mercaptoethanol added to a defined maturation medium improves blastocyst formation or porcine oocytes after intracytoplasmic sperm injection. *Theriogenology*, **65**: 1191-1199.

Koonjaenak, S., P. Pongpeng, S. Wirojwuthikul, A. Johannisson, A. Kunavongkrit and H. Rodriguez-Martinez. 2007. Seasonality affects post-thaw plasma membrane intactness and sperm velocities in spermatozoa from Thai AI swamp buffaloes (*Bubalus bubalis*). *Theriogenology*, **67**: 1424-1435.

Krishnakumar, S., D.P. Whiteside, B. Elkin and J.C. Thundathil. 2011. Evaluation of an animal protein-free semen extender for cryopreservation of epididymal sperm from North American bison (*Bison bison*). *Theriogenology*, **76**: 252-260.

Koning, A.M.H., M.A.Q. Mutsaerts, W.K.H. Kuchenbecher, F.J. Broekmans, J.A. Land, B.W. Mol and A. Hoek. 2012. Complications and outcome of assisted reproduction technologies in overweight and obese women. *Hum. Reprod*., **27**: 457-467.

Lessard, C., J. Danielson, K. Rajapaksha, G.P. Adams and R. McCorkell. 2009. Banking North American buffalo semen. *Theriogenology*, **71**: 1112-1119.

Li, J., H. Zhang, D. Wang, B. Tang, C. Chen, D. Zhang, M. Zhang, J. Duan, H. Xiong and Z. Li. 2011. Rice omics and biotechnology in China. *Plant Omics*, **4**: 302-317.

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

Liang, Y.Y., T. Phermthai, T. Nagai, T. Somfai and R. Parnpai. 2011. In vitro development of vitrified buffalo oocytes following parthenogenetic activation and intracytoplasmic sperm injection. *Theriogenology*, **75**: 1652-1660.

Liang, Y.Y., K. Sriratana, T. Phermthai, T. Somfai, T. Nagai and R. Parnpai. 2012. Effects of vitrification cryoprotectant treatment and cooling method on the viability and development of buffalo oocytes after intracytoplasmic sperm injection. *Cryobiology*, **65**(2): 151-156.

Liebermann, J., F. Nawroth, V. Isachenko, E. Isachenko, G. Rohimi and M.J. Tucker. 2002. Potential importance of vitrification in reproductive medicine. *Biol. Reprod*., **67**: 1671-1680.

Lonergan, P. 2007. State-the-art embryo technologies in cattle. *Soc. Reprod. Fertil. Suppl*., **64**: 315-325.

Lu, Y., M. Zhang, S. Lu, D. Xu, W. Huang, B. Meng, H. Xu and K. Lu. 2010. Sex-preselected buffalo (*Bubalus bubalis*) calves derived from artificial insemination with sexed sperm. *Anim. Reprod. Sci*., **119**: 169-171.

McMahon, C.A., J. Boivin, F.L. Gibson, J.R.W.

Fisher, K. Hammarberg, K. Wynter and D.M. Saunders. 2011. Older first-time mothers and early postpartum depression: a prospective cohort study of women conceiving spontaneously or with assisted reproductive technologies. *Fertil. Steril*., **96**: 1218-1224.

Ménézo, Y.J.R., A. Veiga and J.L. Pouly. 2000. Assisted reproductive technology (ART) in humans: Facts and uncertainties. *Theriogenology*, **53**: 599-610.

Misra, A.K., M.M. Rao, R. Kasiraj, N.S. Reddy and H.C. Pant. 1999. Factors affecting pregnancy rate following nonsurgical embryo transfer in buffalo (*Bubalus bubalis*): A retrospective study. *Theriogenology*, **52**(1): 1-10.

Morrell, J.M. and H. Rodriguez-Martinez. 2010. Practical applications of sperm selection techniques as a tool for improving reproductive efficiency. *Veterinary Medicine International*, **2011**(2).

Mukhopadhyay, C.S., A.K. Gupta, B.R. Yadav, I.S. Chauhan, A. Gupta, T.K. Mohanty and V.S. Raina. 2011. Effect of cryopreservation on sperm chromatic integrity and fertilizing potential in bovine semen. *Livest. Sci*., **136**: 114-121.

Neerja, W., K. Neetu and T. Shuchita. 2009. Optization of embryo culture conditions for increasing efficiency of cloning in buffalo (*Bubalus bubalis*) and generation of transgenic embryos via cloning. *Cloning Stem Cells*., **11**: 387-395.

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

Oropeza, A.J., A.F. Rojas, M.A. Velazquez, J.D. Muro, Y.C. Marquez and L.T. Vilanova. 2010. Efficiency of two timed artificial insemination protocols in Murrah buffaloes managed under semi-intensive system in the tropics. *Trop. Anim. Health Prod*., **42**: 1149-1154.

Owiny, O.D., D.M. Barry, M. Agaba and R.A. Godke. 2009. *In vitro* production of cattle x buffalo hybrid embryos using cattle oocytes and African buffalo (*Syncerus caffer caffer*) epididymal sperm. *Theriogenology*, **71**: 884-894.

Palermo, G.D., Q.V. Neri, D. Monahan, J. Kocent and Z. Rosenwaks. 2012. Development and current applications of assisted fertilization. *Fertil. Steril*., **97**: 248-259.

Pasqualotto, F.F., L.M. Rossi-Ferragut, C.C. Rocha, A. Iaconelli Jr. and E. Borges Jr. 2002. Outcome of *in vitro* fertilization and intracytoplasmic injection of epididymal and testicular sperm obtained from patients with obstructive and nonobstructive azoospermia. *J. Urology*., **167**(4): 1753-1756.

Perera, B.M.A.O. 2011. Reproductive cycles of buffalo. *Anim. Reprod. Sci*., **124**: 194-199.

Presicce, G.A., E.M. Senatore, G. De Santis, R. Stecco, G.M. Terzano, A. Borghese and G.J. De Mauro. 2002. Hormonal stimulation and oocyte maturational competence in prepuberal Mediterranean Italian buffaloes (*Bubalus bubalis*). *Theriogenology*, **57**: 1877-1884.

Pablico, S. 2008. Bright prospect of biotechnology at PCC [Philippine Carabao Center]. *Agriculture*, **1**: 24-27.

Rasul, Z., N. Ahmad and M. Anzar. 2001. Changes in motion characteristics, plasma membrane integrity, and acrosome morphology during cryopreservation of buffalo spermatozoa. *J. Androl*., **22**: 278-283.

Rasul, Z., M. Anzar, S. Jalali and N. Ahmad. 2000. Effect of buffering systems on post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. *Anim. Reprod. Sci*., **59**: 31-41.

Rodriguez-Martinez, H. 2012. Assisted reproductive techniques for cattle breeding in developing countries: A critical appraisal of their value and limitations. *Reprod. Domest. Anim*., **47**: 21-26.

Sansone, G., M.J.F. Nastri and A. Fabbrocini. 2000. Storage of buffalo (*Bubalus bubalis*) semen. *Anim. Reprod. Sci*., **62**, 55-76.

Santos, S.S.D., F.C. Biondi, M.S. Cordeiro, M.S. Miranda, J.K. Dantas, J.R. Figueiredo and O.M. Ohashi. 2006. Isolation, follicular density, and culture of preantral follicles of buffalo fetuses of different ages. *Anim. Reprod. Sci*., **95**: 1-15.

Saragusty, J. and A. Arav. 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction*, **141**: 1-19.

Schippert, C., P. Soergel, I. Staboulidou, C. Bassler, S. Gagalick, P. Hillemanns, K. Buehler and G.J. Garcia-Rocha. 2012. The risk of ectopic pregnancy following tubal reconstructive microsurgery and assisted reproductive technology procedures. *Arch. Gynecol Obstet*, **285**: 863-871.

Schwartz, D. and M.J. Mayaux. 1982. Female fecundity as a function of achieving a first ongoing pregnancy. Because in many Western age, results of artificial insemination in 2193 nulliparous women with developed countries ever more women are desiring to have azoospermic husbands. *New Engl. J. Med*., **306**: 404-406.

Seidel, G.E. Jr. 2009. Sperm sexing technology-the transition to commercial application. *Theriogenology*, **71**: 1-3.

Sharpe, J.C. and K.M. Evans. 2009. Advances in flow cytometry for sperm sexing. *Theriogenology*, **71**: 4-10.

Singh, B., M.S. Chauhan and S.K. Singla. 2009. Reproductive biotechniques in buffaloes (*Bubalus bubalis*): status, prostects and challenges. *Reprod. Fert. Develop*., **21**: 499-510.

Stringfellow, D.A., M.D. Givens and J.G. Waldrop. 2004. Biosecurity issues associated with current and emerging embryo technologies. *Reprod. Fert. Develop*., **16**: 93-102.

Su, L., S. Yang, X. He, X. Li, J. Ma, Y. Wang, G.A. Presicce and W. Ji. 2012. Effect of donor age on the developmental competence of bovine oocytes retrieved by ovum pick up. *Reprod. Domest. Anim*., **47**: 184-189.

Sukhato, P., S. Thongsodseang, A. Utha, N. Songsasen. 2001. Effects of cooling and warming conditions on post-thawed motility and fertility of cryopreserved buffalo spermatozoa. *Anim. Reprod. Sci*., **67**: 69-77.

Sueldo, C.M., A.A. Milki and R.B. Lathi. 2012. Successful frozen blastocyst transfers after failed fresh transfers in assisted reproduction technologies with hydrosalpinx. *American Journal of Obsterics and Gynecology*, **206**: e4-e6.

Tatham, B.G., T. Feehan and R. Pashen. 2003. Buffalo and cattle hybrid embryo development is decreased by caffeine treatment during *in vitro* fertilization.

Theriogenology, **59**: 709-717.

Uhm, S.J., M.K. Gupta, T. Kim and H.T. Lee. 2007. Expression of enhanced green fluorescent protein in porcine-and bovine-cloned embryos following interspecies somatic cell nuclear transfer of fibroblasts transfected by retrovirus vector. *Mol. Reprod. Dev.*, **74**: 1538-1547.

Underwood, S.L., R. Bathgate, M. Ebsworth, W.M.C. Maxwell and G. Evans. 2010. Pregnancy loss in heifers after artificial insemination with frozen-thawed, sex-sorted, re-frozen-thawed dairy bull sperm. *Anim. Reprod Sci.*, **118**: 7-12.

Vajta, G. and M. Gjerris. 2006. Science and technology of farm animal cloning: state of the art. *Anim. Reprod. Sci.*, **92**: 211-230.

van Wagtendonk-de Leeuw, A.M. 2006. Ovum pick up and *in vitro* production in the bovine after use in several generations: A 2005 status. *Theriogenology*, **65**: 914-925.

Vecchio, D., G. Neglia, A. Bella, P. Rossi, E.M. Senatore, G.A. Presicce and B. Gasparrini. 2012. Seasonal effect on pregnancy rates and early embryonic mortality in Pluriparous Mediterranean buffaloes (*Bubalus bubalis*) following artificial insemination with sexed semen. *Reprod. Fert. Develop.*, **24**: 117-117.

Verma, V., S.K. Gautam, P. Palta, R.S. Manik, S.K. Singla and M.S. Chauhan. 2008. Development of a pronuclear DNA microinjection technique for production of green fluorescent protein-expressing bubaline (*Bubalus bubalis*) embryos. *Theriogenology*, **69**: 655-665.

Walker, S.K., K.M. Hartwich and R.F. Seamark. The production of unusually large offspring following embryo manipulation: Concepts and challenges. *Theriogenology*, **45**: 111-120.

Wani, N.A. 2011. Cloning by somatic cell nuclear transfer (SCNT) in Camelids. *The 8th Annual Conference of the Asian Reproductive Biotechnology Society (abstract).* Guilin City, Guangxi, China.

Willadsen, S.M. 1986. Nuclear transplantation in sheep embryos. *Nature*, **320**: 63-65.

Wilmut, I., A.E. Schnieke, J. McWhir, A.J. Kind and K.H. Campbell. 1997. Viable offspring derived from fetal és adult mammalian cells. *Nature*, **385**: 810-813.

Wrathall, A.E., H.A. Simmons, D.J. Bowles and S. Jones. 2004. Biosecurity strategies for conserving valuable livestock genetic resources. *Reprod. Fert. Dev.*,**16**: 103-112.

Wu, X., H. Ouyang, B. Duan, D. Pang, L. Zhang, T. Yuan, L. Xue, D. Ni, L. Cheng, S. Dong, Z. Wei, L. Li, M. Yu, Q.Y. Sun, D.Y. Chen, L. Lai, Y. Dai and G.P. Li. 2012. Production of cloned transgenic cow expressing omega-3 fatty acids. *Transgenic Res.*, **21**: 537-543.

Yilmaz, N., D. Uygur, M. Dogan, E. Ozgu, B. Salman and L. Mollamahmutoglu. 2012. The effect of follicular antimullerian hormone levels of non-obese, non-hyperandrogenemic polycystic ovary syndrome patients on assisted reproduction outcome. *Gynecol. Endocrinol.*, **28**: 162-165.

Zhou, H. and H. Zhang. 2005. Effects of growth factors on in vitro development of caprine preantral follicle oocytes. *Anim. Reprod. Sci.*, **90**: 265-272.