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Modern biotechnological tools for enhancing reproductive efficiency in livestock

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Abstract

Farm animals, an important component of agriculture in Asia and other countries of the world plays a pivotal role in agricultural economy in the form of meat, milk and drought power. This important animal resource needs to be genetically improved owing to considerable dependence of the state economy, food chain as well as the farmer's security upon them. The limitation to genetic improvement imposed by inherent biological factors can be overcome by the use of reproductive bio-techniques which aim to manipulate reproduction - related events and/or structures to achieve pregnancy with the final aim of producing healthy offspring in the females. Assisted Reproductive Techniques (ART) manipulate structures and/or events related to ovulation, fertilization and embryo transfer. It corners around manipulation of female/male reproductive tract physiology, biochemistry, health and thereby longevity. Recently developed bio-techniques used for improving reproductive efficiency of farm animals include artificial insemination, gamete and embryo freezing, multiple ovulation and embryo transfer technologies, *in vitro* embryo production, ovum pick-up, cloning by nuclear transfer (NT) of embryonic and somatic cells, stem cell biology and transgenics. Use of these techniques could enable scientists and researchers to understand and overcome the key issues responsible for limiting full utilization of buffalo to improve the economy.

Key words: Assisted reproductive techniques, farm animals, artificial insemination, *in vitro* fertilization, embryo transfer, embryo sexing, cloning

Introduction

Certain factors associated with reproduction limit the efficient utilization of farm animals, especially cattle, buffalo and goat like late maturity, poor estrus expression in summer season, low reproductive efficiency and fertility rates due to environmental stress coupled with poor management by the farmers in our country. Therefore, considerable attention has been focused to understand the causes responsible for limitations in reproduction by studying their physiology, reproductive endocrinology as well as developing bio-techniques to enhance their genetics and reproductive efficiency. The development of modern procedures like microscopy, centrifugation, ultrasonography, cryopreservation, endoscopy, flow cytometry and sorting, microinjectors, micromanipulators, PCR, electroporators and nucleofectors, brought with them the era of novel, more improved, more efficient, more robust, more reliable and more reproducible techniques known collectively as Assisted Reproductive Techniques (ARTs). ARTs, in the current scenario, include artificial insemination (AI), multiple ovulation and embryo transfer (MOET), trans-vaginal ultra-sound guided oocyte recovery (TVOR), also known as Ovum pick up (OPU), *in vitro* fertilization (IVF), *in vitro* production of embryos (IVEP), cloning, cryopreservation of gametes and embryos, transgenesis, xenografting, germ-cell transplantation, pre-implantation genetic diagnosis and sperm and embryo sexing. Its existence, sustenance and success pillars upon the way the farm animals are/and would be used for meat and milk production, for rapid multiplication of elite germplasm, for germplasm conservation, for multiplication of otherwise naturally incompetent but highly valuable animals, biopharming and the more recent and rightly much hyped endangered species (animal) conservation. Besides, its use in production, its importance lies in studying of reproductive processes to enhance the understanding and knowledge for the most mysterious and subtle part of life- "Reproduction", which is both the key to

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growth and backbone of animal economy. This review provides an overview of assisted reproductive techniques and also of the research done to date towards the enhancement of reproductive efficiency in the farm animals through embryo biotechniques.

Historical background

The history of assisted reproduction may be traced back to seventeenth century when Anton Von Leeuwenhoek first observed sperm under microscope and called them as, "animalcules". Thereafter, Lazzaro Splanzinni (1799) proposed that the contact between egg and sperm was necessary for the embryo to develop and grow. He further carried artificial insemination in dogs and succeeded in getting live births, and went on to inseminate frogs and fish, thereby establishing artificial insemination as the successful assisted reproductive technique. Spllanzini also led to the development of cryobiology with some of his early experiments where he succeeded in keeping the frog, stallion and human sperms viable after cooling in snow and re-warming. It was finally John Hunter, who reported AI in humans, by collecting the spermatozoa from a patient who suffered from hypospadias and injected it into his wife's vagina with a warm syringe, resulting in the birth of a child in 1785. Ivanoff reported AI in domestic farm animals, dogs, foxes, rabbits and poultry. He further developed semen extenders, initiated sperm freezing, which subsequently lead to foundation for the establishment of artificial insemination as a veterinary breeding technique. Around the same time (1890s), the first successful mammalian embryo transfer was performed by Walter Heape, by transferring two four-cell stage Angora rabbit embryos into an inseminated Belgian doe which subsequently led to birth of four Belgian and two Angora kit (Betteridge 2003).Thereafter, Alan Brachet succeeded in keeping alive the rabbit blastocyst in blood plasma for 48 hours. This was followed by establishment of successful pregnancies with embryos obtained after flushing from a number of species from mice and rabbits to sheep and cow. This embryo flushing and transfer became a routine in domestic animal breeding during 1970s. Driven by the potential economic gains and commercial success, a range of assisted reproductive techniques have been developed for farm animal reproduction, which form the subject matter of this paper. Prof. Wilmut from UK started initial works on animal cloning and set the stage ripe for animal science scientists to plunge into the era of cloning, by successfully producing Dolly- the first cloned animal in 1986. Thereafter, multiple assisted reproductive techniques, which are elsewhere dealt with in the draft, were established and their procedures standardized, to bring glory, name, fame and money to the science of assisted reproduction.

Drivers for assisted reproduction

The main driver for the robust development of mammalian assisted reproductive techniques is the considerable plasticity of the mammalian oocyte and embryo. Mammalian oocyte is able to mature, face fertilization and develop into an embryo even in suboptimal conditions, as in *in vitro* environment. Such ability has encouraged, over the decades, the development of numerous *in vitro* assisted reproductive techniques in several species, including humans. The use of ART has further increased because of its perceived safety both to the offspring and the mother (Grossfeld et al. 2005). With the increasing intensity of infertility in humans (15% in reproductive age couples), coupled with the need of the deeper knowledge about pre-implantation development and embryo genetics, the evaluation of the overall ART safety has become a matter of great concern. This makes crucial the studies on pre-implantation embryo development in order to gain insights into the molecular mechanisms responsible for improved, efficient, safe and healthy assisted reproduction. Since, there is an obvious scarcity of human embryos for research; the use of appropriate animal models provides a reliable, cost- effective and irreplaceable support, thereby boosting the assisted reproductive intervention in farm animals, which considerably feature the characteristics of human fertilization. The economic gain and potential of commercial exploitation of these techniques in farm animals' added further oil to the wanting and haunting fire of humankind to drive the era of assisted animal reproduction into the boom.

Assisted reproductive techniques

Under current scenario, the assisted reproductive techniques are classified into four generations (Bertolini and Bertolini 2009) which include: 1) **First generation ART:** The first generation ART includes artificial insemination (AI) and gamete and embryo freezing, 2) **Second generation ART:** These include multiple ovulation and embryo transfer (MOET) technologies, 3) **Third generation ART:** These include *in vitro* fertilization (IVF) procedures. These techniques have matured into successful commercial applications, facilitating the increase in production through genetics, the reduction in generation intervals, the control of

diseases, and the cutback in production costs. Additional techniques that have evolved as different variants of IVF include gamete intra fallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), and intacytoplasmic sperm injection (ICSI), and 4) **Fourth generation ART:** These include processes like cloning by nuclear transfer (NT) of embryonic and somatic cells, stem cell biology and transgenesis and such techniques which are still more experimental.

Despite of the generational classification, it becomes imperative to mention that all these techniques are intertwined and are completely inter-dependent. For example, we need cryopreserved spermatozoa (first generation ART) for IVF (third generation ART) to produce stem cells or to develop transgenic animals (fourth generation ART). It has been observed that the most consolidated reproductive techniques that have been genetically relevant in the past five decades involve mostly the first three generations of ART, primarily AI, cryopreservation of gametes and embryos, induction of multiple ovulations, ultrasonography, *in vitro* fertilization and embryo transfer. The third and fourth generation ARTs, have the potential of enhancing the influence of superior animals on production but their commercial adaptability, as of now, is limited. These ART include sexed semen or embryos, cloning, transgenics, stem cell biology and molecular diagnostics.

From Artificial Insemination (AI) to stem cell biology

Artificial Insemination

AI, the first generation ART, has been in use for the last 200 years. It has been the most successful and efficient reproduction technology in animal production for the last six decades, owing to successful achievements in semen cryobiology. AI has now become a practical technology in commercial dairy cattle programs in both developed as well as developing countries. The real revolution in AI came when frozen semen was used by Polge and his group (1949), which led to efforts to freeze buffalo semen until 1972. AI was initially used to spread improved indigenous breeds, which was followed by the more valuable crossbreeding. This main advantages of AI as an assisted reproductive technique in cattle and buffalo are: rapid dissemination of superior genetic material, maximizing the use of superior males, greater efficiency and rate of genetic selection, introduction of new genetic material by import of semen rather by cumbersome import of the live animals, considerable reduction in transportation and maintenance hurdles, and reduced risk of sexually transmitted diseases. It further enables animal scientists to exploit the progeny tested bulls, even after their death, by using the frozen semen. Since, male animals are larger than females and thus require relatively more space and food, AI enables a farmer to save the costs required for maintenance of more strong, powerful and potentially ill-mannered male animals, thus saving the costs incurred in special housing and handling equipments. Such is the impact of AI that 50% of estimated increase in milk production in developed countries during the second half of the twentieth century, is attributed to genetic gain obtained by wide spread use of AI over conventional breeding (Lohuis 1995).

AI was used for water buffalo in early forties at Allahabad Agricultural Institute in India and the first calf was born in 1943. Currently in India, 67 million frozen semen doses are produced and number of artificial inseminations performed has reached to 54 million, bringing about 22 million animals under AI with an overall conception rate of 35%. The government has planned to initiate a new scheme namely, National project on bovine breeding and dairy, which plans to bring 80% breedable females among cattle and buffalo under organized breeding through artificial insemination or natural service by bulls of high genetic merit. The project also has the mandate to arrange the AI services at the farmers' doorsteps.

Gamete and embryo cryopreservation

It is rightly believed that it was the attainment of successful semen cryopreservation protocols that sustained AI as the most successful and efficient ART. It was frozen semen that boosted the dairy industry by making AI simpler, economical, successful and widespread. One of the most critical requirements for *in vitro* embryo production (discussed ahead) is the continuous availability of viable and developmentally competent oocytes. Thus, considerable efforts were put in to devise the methodology which would enable the researchers to store and preserve the unfertilized oocytes without compromising their developmental potential. During the past few decades significant progress in oocyte cryopreservation has been achieved and live offspring of as many as 25 different species have resulted from transfer of cryopreserved oocytes or embryos (Gajda and Smorg 2009). Like the preservation of its counterpart in AI, preservation of oocytes reduces the risk and expenses involved in

live animal transport, hazards of diseases transmission and natural disasters and accidents. The preservation of oocytes from endangered species also safeguards them from extinction. The major problems viz., mechanical damage and osmotic shock, which were main obstacles in gamete cryopreservation fell apart with the advent of such cryoprotectants like glycerol, ethylene glycol, propylene glycol (permeating cryoprotectants), sucrose, glucose or fructose (non-permeating agents) (Luz et al. 2009; Barcelo-Fimbres and Seidel 2007). The process of vitrification was subsequently shown to be more effective than slow freezing for materials more sensitive to chilling in cattle (Vajta et al. 1996) and buffalo (Gautam et al. 2008). The cryopreservation of oocytes by vitrification was achieved in various species like bovine, swine, equine and buffalo (Hochi et al. 1998; Huang and Holtz 2002; Hurtt et al. 2000; Sharma et al. 2007). The successful embryo cryopreservation allowed the global commercialization of animals of high merit, as embryos. Embryo freezing is an established practice in commercial embryo transfer programmes where at least temporary storage of embryos, until they are transferred, is the essential need as the embryo viability starts declining after 12h in holding media. We succeeded in our laboratory to produce a live calf, 'Shreshth' from transfer of vitrified-warmed blastocysts (Saha et al. 2012). This calf was genetically and physiologically normal and has grown in to an adult bull (Fig. 1). Moreover, the cryobanking of embryos is perceived as a very helpful strategy for conservation of endangered species of animals and thus demands an obvious attention owing to ruthless devastation of the natural habitats.

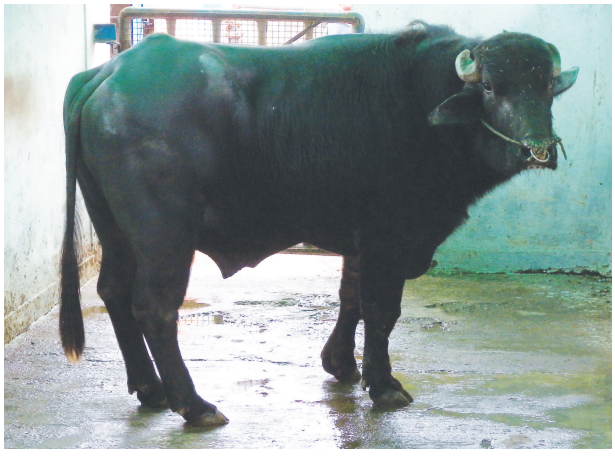

Fig. 1. ***Shreshth*- A male buffalo produced from Embryo transfer of vitrified warm blastocysts developed through Hand-made cloning using buffalo new born fibroblasts as donor cells**

In-vitro embryo production (IVEP)

The *in vitro* production of embryos usually comprises of three steps: 1) *In vitro* maturation (IVM) of primary, germinal-vesicle stage, oocytes collected either directly from the ovaries of donor female animals (ovum pick up) or aspirated from the slaughter-house obtained ovaries; 2) *In vitro* fertilization (IVF) by combining IVM oocytes with *in vitro* capacitated sperm cells; 3) *In vitro* culture (IVC) of presumed zygotes to developmental stages fit for transfer into recipient females. IVEP helps in production of high genetic merit animals, besides providing an excellent source of embryos for other assisted reproduction technologies like cloning, transgenesis, embryo sexing, stem cell research etc. IVEP also provides sufficient embryos to be used for studying developmental and embryo genetics, proteomics, epigenetics as well as cytogenetic disorders (Galli and Lazzari 2008). The speculation that early stages of bovine embryo development may show similarities with human embryos makes bovine embryos as model organisms (Niemann and Wrenzycki 2000) and thus IVEP as a tool to generate the requisite raw material. The major thrust area in buffalo IVEP is to improve its efficiency which is currently around 30% to 40% well below the expectations (Sirard et al. 2006).

Despite several advantages of this technique, its initial application in cattle and buffaloes was limited (Chauhan et al. 1998) primarily due to scarcity of the oocytes from these species. However, the recent developments of transvaginal oocyte retrieval (TVOR) and ovum pick up (OPU) have removed these difficulties to a large extent. We have reported the success of this technique in giving birth to India's first female Sahiwal calf named 'Holi' from aged animal (Saini et al. 2015) (Fig. 2). Also, the standardization of procedures for buffalo IVM, IVF and IVC have been instrumental in thriving the IVEP in the poor breeding bubaline species (Chauhan et al. 1997, 2005). The greatest limitation of IVEP is the high production costs and the low overall efficiency under field conditions (Palta and Chauhan 1998). Despite the hopes generated by IVEP, it remains still unclear whether IVEP provides a reliable alternative to conventional super ovulation and embryo transfer for producing embryos from reproductively healthy buffaloes.

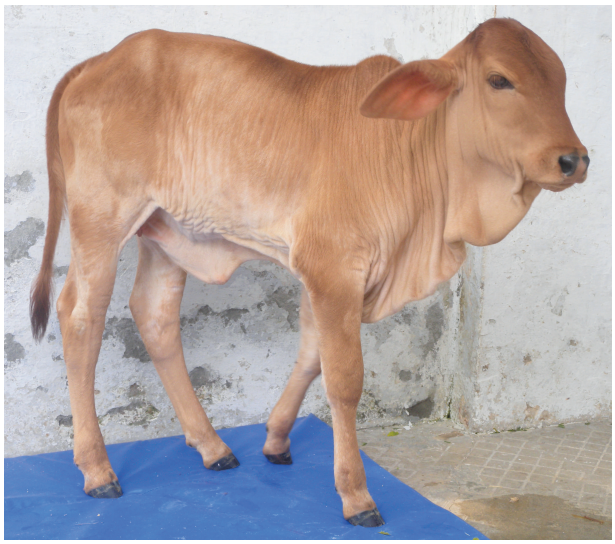

Fig. 2. A female calf named *Holi* produced from aged *Sahiwal* cow through IVF-OPU technology

Semen and embryo sexing

Predetermination of the sex of offspring would inevitably lead to selective multiplication of the desired sex. It is presumed that known sex of embryos produced for use in ET programs can more effectively help to manage producer resources, because it would enable to produce more heifer calves per ET, which is the main goal of the dairy entrepreneur. The presence and absence of Y elements determines the sex of embryo or semen and currently this is determined by: 1) chromosomal analysis of demioocytes; 2) immunological detection of embryonic H-Y antigen; 3) fluorescent *in situ* hybridization; 4) Y-specific probes; 5) loop mediated isothermal amplification reaction and 6) flow sorting of semen (Garner and Seidel 2008; Zoheir and Allam, 2010). It has been reported that bovine Y-specific sequences are conserved among buffalo, Indian *Zebu* and *Tarus* cattle. Thus, the use of bovine Y-specific probes/primers would be used to demonstrate the sex of buffalo or zebu embryos. Embryo biopsy method is also used in which a single cell from early stage embryo is extracted and probed for presence or absence of Y chromosome (Lopatarova et al. 2008). The offspring of desired sex have already been produced employing flow sorting for sexing of both fresh and frozen-thawed semen in several species like cattle, goat, pigs and sheep (Seidel et al. 1999; Parrilla et al. 2004; Grossfeld et al. 2005; De Graaf et al. 2007). In case of buffalo, birth of the first buffalo calves produced by the combined use of AI and sexed semen has been reported by Presicce et al. (2005). The semen and embryo sexing has not been reported in field in any developing country except China. In India the refinement of the techniques of sperm and embryo sorting is currently limited to research institutions only.

Embryo transfer

Embryo transfer (ET) is a technique in which embryos are collected from a donor female and transferred to recipient females which serve as surrogate mothers for the remainder of pregnancy. It has been used to increase the reproductive performance of particular females of agriculturally important species like cattle, horse, sheep and goat. The commercial ET started wayback in 1970s in North America primarily as a means of multiplying the number of young by exotic breeds of beef cattle. Then the advent of non surgical embryo recovery and transfer methods, lead to the expansion of this technique beyond the restricted domains of the surgical means of embryo recovery and transfer.

The initial success of embryo transfer in riverine buffalo in the US (Drost et al. 1983) was followed by the birth of calves in Bulgaria (Vlanovet al. 1985) and India (Misraet al. 1994). In 1991, a riverine buffalo calf (2n=50) was born out of transfer in a swamp buffalo recipient (2n=48) (Cruz et al. 1991). The other crucial requirement for commercial exploitation was completed by the effective freeze-thaw method which permitted embryos to be shipped anywhere to the world. ET or MOET (multiple ovulation and embryo transfer) offers various commercial advantages in like: i) easy and affordable transport of the desired embryos; ii) genetic improvement in domestic animal industry by obtaining a large number of desirable progeny from elite parents; iii) high quality breeding males to be available for sale; iv) developments in breeding technology like embryo sexing and embryo-splitting; v) utilization of the combined genetic contribution of the male and female at the same time; vii) production of artificial insemination sires from highly proven cows and bulls. The success of MOET program has lead to the use of this technology to test AI sires genetically.Under this program selected buffaloes are super stimulated and inseminated to highly proven bulls. The male offspring are placed in waiting while female offspring are used for ET. The bulls are then proven by production records from siblings, rather than by progeny. Using this approach it is possible to test the bull genetically within three and a half years as compared to five and a half years using traditional progeny testing schemes.

Pregnancy rates following non- surgical transfer of *in vivo* derived buffalo embryos was very low in early trials except for the 100% success achieved by Drost et al. in transferring a single embryo in 1983.

Although the basic procedures employed in buffalo for ET are well established, considerable research is required for further improvement with the main focus on recovery of oocytes from live females by ultrasound guided aspiration and in subsequent IVF and IVC.Also at this time the technical costs involved in ET/MOET preclude its other applications but for seed-stock production, so re-consideration of the economics of ET is the need of the hour. ET and the associated techniques have been utilized for the rapid multiplication of the elite breeds of cattle, buffalo, sheep, goat, horse and pig (Madan et al. 1993; Holm et al. 1996; Pawshe et al. 1994; Seidel et al. 1999; Squires et al. 1999).

Cloning

Cloning represents the fourth generation of ART that has the potential to be used for multiplication of elite animals and minimization of the genetic variation in the experimental animals, besides being used for conservation and propagation of endangered species. Somatic cell cloning (SCNT) also offers opportunities to select and propagate animals of specific merit or of desire. Cloning can also be used as a tool in therapeutics for generation of the autologous stem cells. Numerous types of cells are used as donors in cloning, viz., fetal fibroblasts, adult fibroblasts, granulosa cells, hepatocytes, lymphocytes (Campbell et al. 2007). Wilmut et al. (1997) produced the first cloned animal-Dolly and paved the way for the application of this assisted reproductive technique to almost every species like cattle, pig, goat and horse (Cibelli et al. 1998; Polejaeva et al. 2000; Baguisi et al. 1999; Galli et al. 2003). A survey conducted by OIE in 2005 regarding the cloning technique, revealed that 91 countries, comprising especially the developing countries, have the cloning capabilities (MacKenzie 2005). Among the participating countries 60% were developing countries, 4% were from Africa, while 23% of the respondents were from Asia. Recently, the first cloned camel named, Injaz, a female, was born in 2009 and Bin Sougham, a male, was born in 2010 at the Camel Reproduction center in Dubai, United Arab emirates. Also the world's first buffalo calf named Garima was born at National Dairy Research Institute, Indian in 2009 by utilizing an in-house developed technique of Hand- made cloning (HMC). Later on by utilizing the same technique, Garima-II, Purima and Lalima were also produced at the same institute, using buffalo embryonic stem cells and adult fibroblasts as the donor cells (Fig. 3).

Fig. 3. *Garima* II with her calf *Mahima*. Garima II was produced by Hand-made cloning using buffalo embryonic stem cell as donor cell, born on 22nd Aug, 2010. *Mahima* was produced through AI and was delivered via normal parturition on 25 Jan 2013

Reproductive cloning holds the promise of bypassing the conventional breeding procedures to allow development of the duplicates of the genetically engineered animals. It is believed that cloning could be used in future in xenotransplantation to produce "humanized pig", the organs of which could be transplanted to humans. Cloning is also used efficiently for production of transgenic animals by employing several biotechnological techniques like pro-nuclear microinjection, cytoplasmic microinjection, retrovirus- and lentivirus- based vectors, nucleofectors, electroporators, etc. These transgenic founder animals, once produced, could be used both in breeding and biomedicine (Wilmut et al.1997). There is currently a great interest in developing transgenic cows producing recombinant proteins in mammary gland which could be ultimately harnessed through milk.

Stem cell technology and transgenic animal production

An extensive work has been carried out in embryonic stem cell technology, spermatogonial stem cell technology and transgenic research in goat and buffalo, especially at National Dairy Research Institute, Karnal. The culture systems have been developed for propagation and maintenance of buffalo and goat embryonic stem cells as well as testicular stem cells.

We (Shah et al. 2015 a and b) also reported the propagation of bubaline embryonic stem cells for more than 100 passages as well as the potential of these cells to differentiate into all three germ layers (ectoderm, mesoderm and endoderm). Signal transduction and different differentiation strategies have been performed in embryonic stem cell and culture systems have been developed to induce their differentiation into many cell types including germ cells (Shah et al. 2016, 2017). Spermatogonial stem cell culture strategies have been developed and the work for production of transgenic buffalo and goats is currently being carried out and it is hoped that the transgenic animal will in a short time period become a practical reality.

Future strategies

The procedures for IVEP *viz*., IVM, IVF and IVC have to be redesigned or modified to increase the efficiency of embryo formation in cattle, goat and buffalo. This might be alleviated to some extent by introduction of sequential media, which would specifically suffice the nutritional and other requirements depending on the developmental stage. The placental incompatibility between the embryo and the surrogate mother has to be overcome in order to increase the efficiency of cloning and embryo transfer. The growing interest for producing transgenic founder animals would dominate the future pharmaceutical industries, and this consequently warns better understanding of SCNT cloning, in terms of both embryonic genetics and epigenetics. Embryo genomics has to be given the desired attention in order to elucidate the genetics of abnormal embryo production by cloning and other ARTs as well for understanding the embryonic defects at cellular level. One of our prime future strategies should be the introduction of stem cells and nanotechnology and their integration with other ARTs to produce such animals which may contain all desired characters.

Declaration

The authors declare no conflict of interest.

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