Innovative Reproductive Technology in Animal Breeding: A Review

Abstract

The profitability and the intensity achieved by the genetic improvement is determined by the reproductive performance of the farm animals. The major cause of economic loss is reproductive inefficiency in the livestock sector. In the past few years, several advances has been made in reproductive technologies (RTs) to increase the efficiency of genetic improvement. These technologies involves- Artificial Insemination, Sexed semen Technology, Cryopreservation, Multiple Ovulation and Embryo Transfer, In-vitro Embryo Production (IVEP), Transgenesis etc. The problems of infertility of male or female, postpartum infertility in high yielding animals of high genetic merit individuals and breeding of distant species has been overcome through these technologies. The inclusion of these techniques in animal breeding had made possible to obtain large number of progenies from genetically superior animals. This review, discuss the various innovative reproductive technologies implemented in livestock sector.

Keywords: Estrus Detection, Artificial Insemination, Sexed semen Technology, Cryopreservation, Multiple Ovulation and Embryo Transfer, In-vitro Embryo Production technology, Transgenesis

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Introduction

In 2023, the estimated total population in India amounted to approximately 1.43 billion. The improvement in the genetic field of the livestock animals is a major concern over these years. The need for innovative reproduction technologies in livestock animals arises from the desire to improve productivity, health and genetic diversity within livestock populations. By enhancing these technologies, farmers can increase the efficiency of breeding and production, leading to a greater supply of food for the growing human population. RTs can be used to select for and produce livestock with desirable traits, such as high milk production in cows or disease resistance in poultry. This can lead to better quality and quantity of animal products, such as milk, eggs, and meat, which are important source of nutrition for the human population. These technologies can help address challenges such as genetic disorders, low fertility rates, and limited access to superior breeding stock. By using advanced RT, farmers and breeders can more effectively manage genetic traits, improve the health and welfare of animals, and ultimately enhance the overall efficiency and sustainability of livestock production.

To achieve this, several reproduction technologies are engaged in livestock breeding programme. These technologies play an important role that enables to increase genetic gain. Innovative reproduction technologies in livestock animals have been developed to improve breeding efficiency, genetic selection and overall productivity in livestock industry. The major reproduction technologies that are currently used by livestock breeding sectors are Estrus Detection Artificial Insemination (AI), Sexed semen technology, Cryopreservation, Multiple Ovulation and Embryo Transfer (MOET), In-vitro Embryo transfer Technology and Transgenesis. These technologies alone or in combination with other techniques are

implemented in livestock breeding procedures for efficient production of genetically superior offspring from genetically superior breeds. Additionally, these technologies can play crucial role in conserving rare and endangered breeds, ensuring their survival for future generations.

1. Estrus Detection

The behavioural changes and the physiological signs exhibited by the dairy cows before the ovulation period is referred as Estrus (Mičiaková *et al*. 2018). It is the period during the oestrus cycle when the female animals are ready to mate or sexually receptive for mating to become pregnant (Riaz *et al.* 2023). The behavioural changes occur due to the changes in the estrogen and progesterone levels (Paterson *et al.* 1992). Cows in estrus are more likely to mount other cows or to be mounted by other cows (Roelofs *et al.* 2010). Several secondary signs are being exhibited by the cows in estrus such as increase in movement, reduction in feed and water consumption, swelling of vulva, reddening of vulva, mucus discharge from vulva, sniffing in the genitalia area (Röttgen *et al.* 2018). Different devices are developed eventually for detection of estrus in female animals such as pedometer and accelerometer (Silper *et al.* 2015). Recently, a new device has been emerged for detection of estrus *viz* infrared thermography (Marquez *et al.* 2021). Infrared thermography cameras are very efficient in detecting minute changes in body temperatures of livestock animals (Nääs *et al.* 2020). To acertain the appropriate timing for artificial insemination, accuracy and efficient estrus detection is important (Endo 2022).

Table no. 1: Studies on application of Infrared Thermography (IRT) to detect estrus in cattle and

Buffalo (Riaz *et al.* 2023)

Sr. no.	References	Livestock animals	Site of Infrared thermography observations
1.	Talukder et. al., 2014	Dairy cows	Vulva and muzzle
2.	Marquez et. al., 2019	Dairy cows	Eye, muzzle, cheek, neck, front right foot, front left foot, rump, flank, vulva area, tail head, and withers
3.	Marquez et. al., 2021	Dairy cows	Vulva
4.	Rajput et. al., 2022	Sahiwal cows	Vulval, eyeball, ear and muzzle
5.	Tiwari et. al.,2022	Sahiwal cows	Muzzle and Vulva
6.	Marquez et. al., 2022	Dairy cows	Vulva

2. Artificial Insemination

Artificial Insemination is the first-generation tool used in Reproductive Biotechnology (Bharali and Sonowal 2019). AI is a reproduction technology in which semen is manually placed inside the vagina of the female animals (Gelayenew and Asebe 2017). This technology is less complex, less invasive, low cost and therefore the most assuring biotechnology for companion animals (Vishwanath 2003). The first successful insemination was performed by Lord Spallanzani in 1984 in Bitch. For preserving and spreading male genetic material (Souza-Fabjan *et al*. 2023) and to utilize genetic material from male semen donors that have aged or

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suffered from fertility-limiting injuries, AI is generally used (Fonseca *et al*., 2019; Souza-Fabjan *et al*., 2021). To increase the production of local livestock animals, AI is performed by utilizing semen from exotic breeds (Verma *et al.* 2012). The success of Artificial Insemination depends on the efficiency of semen collected and preserved and is inclusion of various processes such as collection, Evaluation, Dilution of semen (Semen Extenders), packing (in medium- 0.5 ml and mini straws- 0.25 ml), Semen preservation (cryopreservation), Thawing of semen (Patel *et al.* 2017).

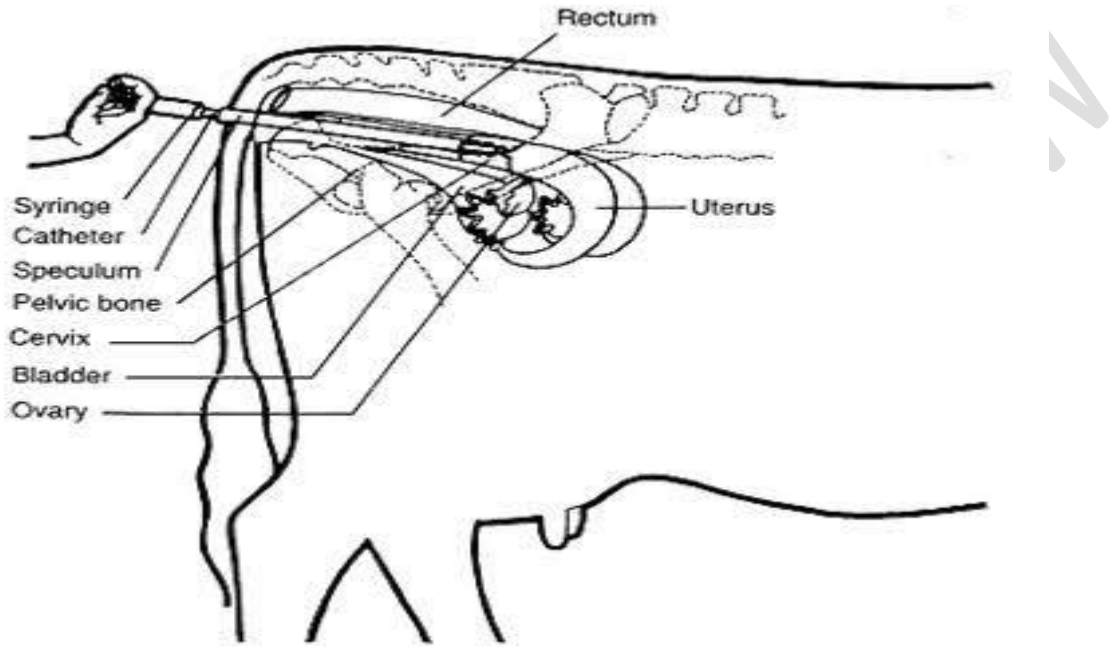

Figure 1: Speculum method for inseminating the cow (Farmers Trend 2014).

3. Sexed Semen Technology

This technology deals with the physical separation of X and Y chromosome bearing spermatozoa in collected semen before inseminating female animals (Joshi *et al.* 2021). Several studies on the semen sexing found that the technology is mostly depend on the DNA content in the X and Y chromosome as X chromosome contains more DNA (3.8%) than Y chromosome (Rahman and Pang 2020; Joshi and Mathur *et al.* 2021).

The significant approaches toward the sperm sexing are the Albumin gradient, the identification of H-Y antigen, the Free-flow electrophoresis, the Detection of sex specific proteins, The Centrifugal counter current distribution. Separation of X and Y bearing spermatozoa with the use of albumin gradient was first time reported by Ericsson *et al.* 1973. The success rate reported through this method of sperm sexing was about 75% (Beernink *et al.* 1993; Kumar *et al.* 2017). Separation of X and Y bearing sperm cell in the identification of H-Y antigen depends upon the specific cell surface antigens on X and Y sperm cells through magnetic bead or affinity chromatography (Hoppe *et al.* 1984; Hendriksen *et al.* 1996; Blecher *et al.* 1999; Hendriksen 1999 and Kumar *et al.* 2017). Yadav *et al.* (2017) reported that H-Y antigen was present in X- chromosome containing spermatozoa as well as in erythrocytes and

premeiotic germ cells and cannot be used for separation of these cells because it does not specifically bind to Y chromosome bearing spermatozoa. Sperm sorting by free flow electrophoresis is done on the basis of the charge (negative by X chromosome and positive by Y chromosome) contained by spermatozoa under electric field conditions (Kaneko *et al.* 1984, Mohri *et al.* 1986 and Kumar *et al.* 2017). Howes and his colleagues in 1997 were the first one to work on the identification of differentially expressed proteins between X and Y spermatozoa but their work was indecisive. In Holstein bulls the differentially expressed proteins in X and Y sperm cells were profiled by Shen *et al*., (2021). The study showed that 8 and 23 surface proteins in X spermatozoa were up-regulated and down-regulated. About 81 and 151 proteins were also reported in the study that were exclusively expressed in X and Y spermatozoa respectively. Various immunological approaches for semen sexting can be developed on the basis of the different sperm types and their proteomes differences. Eventually the separation of X and Y chromosome bearing sperm cells can be achieved using the biomarkers that are developed from the these unique or differentially expressed proteins. The other technique used for sperm sorting is Flow Cytometry. The certainty of Flow Cytometry depends on the DNA contents of the particular chromosome bearing spermatozoa i.e., X- bearing sperm contains 3.8% DNA more than Y- bearing sperm (Johnson 2000). The Flow Cytometry technique of sperm sorting Uses Fluorescent Dyes to stain the DNA in sperm (Caroppo 2013; Ribeiro *et al.* 2013). The stain used for staining the DNA is Hoechst 33342 and is non-toxic, initially sperms are being stained with Hoechst 33342 stain and are then exposed to UV laser beam. After exposing it to the UV beam the emitting fluorescent light is detected and analysed (Johnson and Welch 1999).

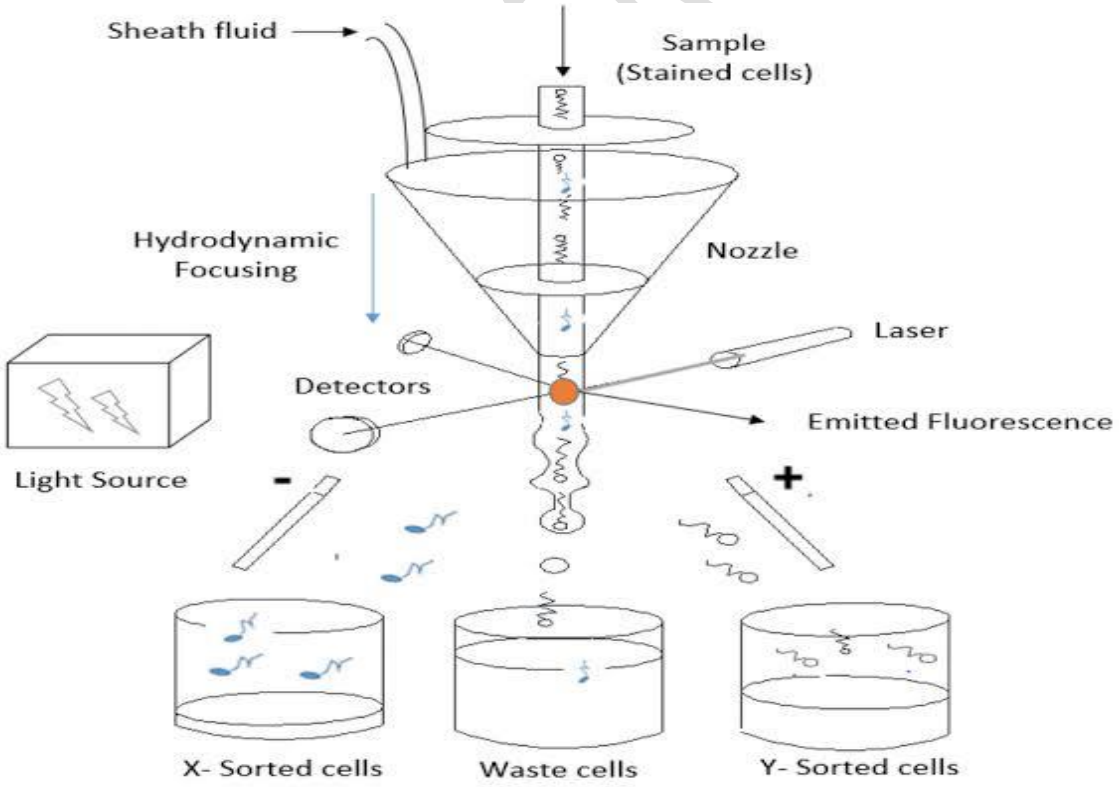

Figure 2: Flow- cytometry based semen sorting (Naniwa et al., 2018).

4. Cryopreservation

There has been considerable success achieved in preservation of semen, oocytes or embryo in farm animals through Cryopreservation (Huang *et al.* 2018). The mating of female cattle that are not bound to time and places can be possible through cryopreservation of semen with minimized risk of disease transmission (Roca *et al.* 2016). The cryopreservation of gametes and embryo can be achieved by two processes (i) slow freezing(programmed) and (ii) vitrification. In slow freezing, the cells or embryos after being treated with cryoprotectants (CPAs) such as glycerol or dimethyl sulphoxide (DMSO) are subjected for slow freezing at the rate of 1ºC per minute by using certain devices such as rate-controlled freezer or a benchtop portable freezing container (Day *et al.* 2007). Vitrification is an ultrarapid cooling process in which gametes and cells are directly placed in CPAs and are immediately drived in liquid nitrogen. Only few minutes are required for the vitrification process if compared with slow freezing process which minimizes the exposure time to sub-physiological conditions (Brambillasca *et al.* 2013). The formation of ice crystal is prevented in vitrification method by rapid cooling and warming rates (Day *et al.* 2007). The cryoprotectants (CPAs) used in cryopreservation are of two types (i) permeating and (ii) non-permeating CPAs. Glycerol and dimethyl sulphoxide are the permeating CPAs, DMSO have high penetrating rate (Saha *et al.* 1996), but can become toxic at higher temperature (Kasai *et al.* 1981). The non-permeating CPAs are Ficol, Sucrose and Trehalose. The addition of these CPAs increases osmotic pressure and are also helpful in penetration of Ethylene Glycol (EG) and DMSO. The study also reported that the freezing tolerance of oocytes can be improved by trehalose (Guo *et al.* 2000; Chen *et al.* 2001; Puhlev *et al.* 2001). Frostie, a Hereford-Friesian was the first calf born from frozen thawed embryo in 1973 (Wilmut and Rowson 1973).

Table no. 2- Milestone achieved in vitrification in embryo (Gordon 2017)

Year	**Species**	**Researcher**
1985	Mouse	Rall and Fahy
1986	Cow	Massip *et al.*
1989	Rabbit	Smorag *et al.*
1990	Sheep/Goat	Scieve *et al.*
1994	Horse	Hochi *et al.*
1998	Pig	Kobayashi *et al.*

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5. **Multiple Ovulation and Embryo Transfer**

Walter Heape (1890) was the first to utilize the technique of Multiple Ovulation and Embryo Transfer. The first embryo transfer in bovine was reported in 1949 by Umbaugh. The development of calf for the first time through embryo transfer was done in 1950 (Willet *et al.* 1951). Earlier the collection and transfer of the embryos was performed surgically through mid-ventral exposure of uterus and ovaries (Genzebu 2015). The non-surgical recovery and transfer of embryos was made possible in mid 1970s with successful cryopreservation of bovine embryo The production of the young ones through the process of MOET involves i) Selection

of Donor cow; ii) Superovulation of Donor cow; iii) Insemination of Donor cow; iv) Development of Embryo (*in-vivo* or *in-vitro)*; v) Embryo recovery (Surgically or Non-surgically); vi) Selection of recipient cow; vii) Synchronization of Recepient cow; viii) Embryo handling, Evaluation and Storage; ix) Embryo Transfer Method (Menta 2023).

5.1 Selection of donor cows

The three important points to consider the selection of donor cow are genetic superiority, reproductive ability and high value of progeny market (Mikkola, 2007; Besenfelder *et al*., 2020). The body condition score should be appropriate at the time of embryo transfer (Habtie 2019). Rather than selecting donor cows, importance should be given to selection of males as males are normally bred to many females and should be selected more accurately. The selection of genetically superior male is very important as 50% of the genetic material is acquired through males, the high-quality semen is especially used from these bulls (David and Hamilton 2016).

5.2 Superovulation of donor cow

The objective of the superovulation is to induce more ovulations than normal rate and can be induced by administrating either PMSG (pregnant mare serum gonadotropin) commonly known as equine chorionic gonadotropin (eCG) or FSH. PMSG is administered on day 10 of the estrus cycle followed by injections of Prostaglandin within 2-3 days at an interval of 12-24 hr. The eCG stimulates greater super ovulatory response than FSH; although good quality of transferrable embryos is obtained through Follicle Stimulating Hormone (FSH) treatment (Selk 2013). The main disadvantage of PMSG is that it causes antibody formation and ovarian cyst in donor animals (Tekeli 2010). FSH is obtained from horse, sheep and pig (Akyol 2001) and is a pituitary gonadotropin. It is produced in the gonadotroph cells located in the anterior lobe of the pituitary gland (Yilmaz 1999), The FSH should be administered in repeated dose because the half-life of Follicle Stimulating Hormone is nearly 2 hours and given two times a day (Kimura *et al.* 2007). The best time to administer FSH is from 9^{th} to 14^{th} days of the estrus cycle (Kanagawa *et al.* 1995).

It is important to maintained hormonal balance through maintained diet as it is necessary for fertilization and embryo development before entering the uterus, at ampulla-isthmus junction (Nicholas and Smith, 1983; Burnett *et al*., 2018). The recipient cow should be disease free for embryo reception and to perform number of pregnancies (Selk 2010). For intrauterine embryo development a well-developed corpus luteum played an important role (Mattos *et al*., 2011; Hansen, 2020). After insemination, the embryos are collected after 7 days from uterus (Habtie 2019).

5.3 Synchronisation of recipient cow

The condition of the reproductive tract of recipient cow should be similar to that of donor cow for the transferring the embryo, for acquiring similar conditions synchronisation of the recipient cow should be done at the same time as donor cow (Galina & Orihuela, 2007). In recipient cow the occurrence of PGF2α-induced estrus is observed after 60-72 hours, therefore the recipient's synchronisation is done 12 hours before the donor cow (Genzebu 2015).

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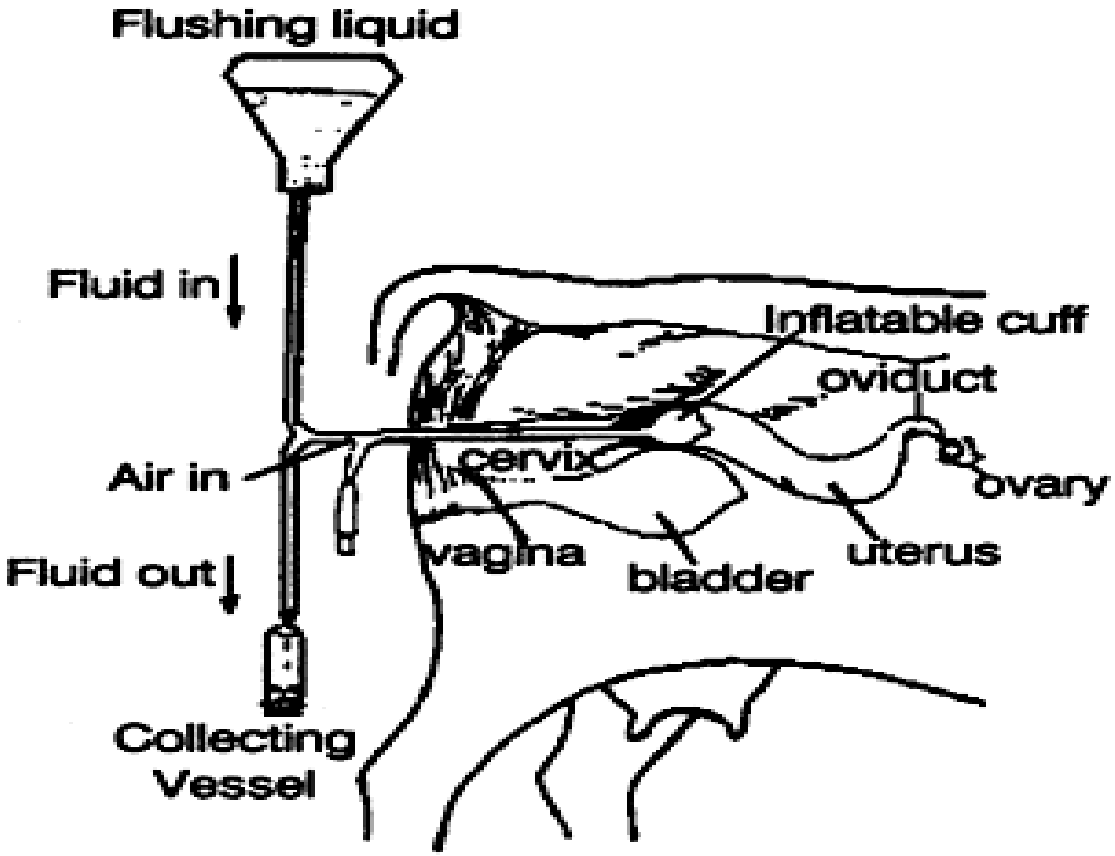

Figure 3: Diagram of the embryo flushing and recovery procedure (Selk, 2010).

6. In-Vitro Embryo Production

The In-vitro Embryo Production process involves three steps- in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro development (IVD) of the resulting embryos (Gelayenew & Asebe 2016). At the time of birth thousands of oocytes are present inside the ovary and are lost due to atresia. The loss of genetic material through atresia can be reduced by harvesting oocytes from ovary followed by IVEP technique (Brakette and Zuelke 1993; Hasler 1998). The first calf produced by in vitro fertilization (IVF) in 1981 and was names "Virgil" (Brakett *et al.* 1982). The main principles of IVEP are selection of donor, selection of recipient animals and the collection of embryos (Kidie 2019). Addition of Nano-Selenium and Nano-Zinc Oxide during oocyte maturation increases maturation rate significantly (Abdel-Halim and Helmi 2017). In vitro fertilisation has also been used to produce the thousands of embryos (Gordon and Lu 1990). IVF through intracytoplasmic sperm injection (ICSI) is prominently used in assisted reproductive technologies (Keskintepe *et al.* 2002). For most cattle farmers this technology is an advantage only for extremely valuable cows that are infertile or fail to respond to superovulation.

6.1 In-vitro Maturation (IVM): Collection of oocytes can be achieved either manually through ultrasound-guided transvaginal follicular aspiration (ovum pick-up) or surgically from the ovaries of slaughtered animals or live animals by mid-ventral exposure of uterus and ovaries. Follicles of 3-5 mm diameter are mostly utilised for oocyte collection. Oocytes with compact cumulus and corona are selected for culture. In-Vitro Maturation of oocytes is conducted for 24 hrs at 38.5^{o}C in presence of 5% CO2 in a CO2 incubator.

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6.2 Preparation of Spermatozoa: Capacitated sperm is essential for in-vitro fertilization. After capacitation, acrosome reaction in the head of sperm allows the release of certain enzymes which help in penetrating the zona pellucida of the oocyte.

6.3 In-vitro Fertilization (IVF): The capacitated sperm and the collected matured oocytes are co-cultured and are allowed to incubate at body temperature of animal for 6-24 hrs.

6.4 In-vitro embryo development: It is a component of IVF where in resultant embryos (Zygote) are allowed to grow for some time in an artificial medium. Culture is continued for 24 to 36 hrs before a further assessment for embryo cleavage is performed. At this stage, the embryos are normally between two to eight cell (blastomere) stages. Embryo quality is assessed depending on blastomere symmetry and the degree of cytoplasm fragmentation before transfer.

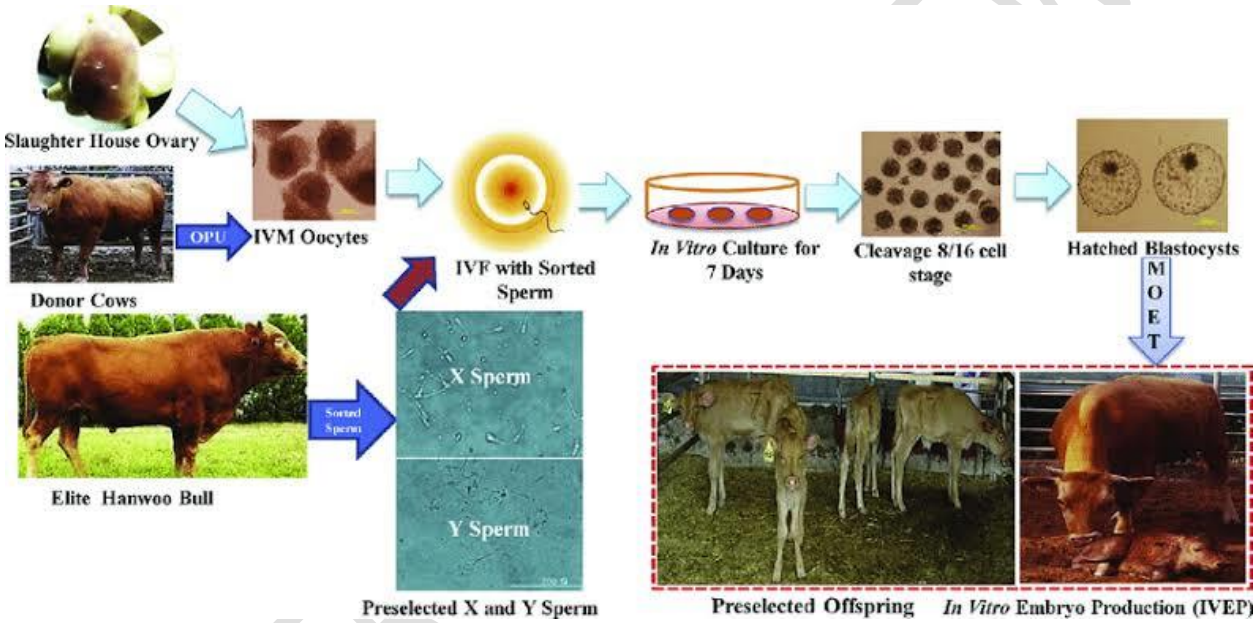

Figure 4: In vitro fertilization and embryonic development (Chowdhury, et al., 2019).

7. Transgenesis

Transgenesis technique involves the manipulation of genes of an organism and deliberately addition of that genome into the genome of organism of same or other species (Shankar and Mehendale, 2014). Palmiter et al., (1982) was the first to use genetic engineering in the improvement of livestock animal. The first success through this technology was obtained in laboratory animal mice by Jaenisch and Mintz in 1974. The first transgenic calf "Rosie" was produced in 1997 (Clark 2002) and was helpful in the production of human protein-enriched milk. It is not possible to make alterations in animal genome through conventional breeding, to fulfil the inadequacies of the conventional breeding programme, transgenesis technique is used. The transgenic animals produced from the process of transgenesis shows great advantages such as Increase in Feed Conversion rates, increase in growth rate of meat animals, increase in muscle mass, improved nutritional quality, Increase in disease resistance animals, Xenotransplantation's etc. Various methods are used for the production of transgenic animals such as DNA microinjection, Use of Transposon's, Retrovirus mediated-gene transfer, Embryonic stem cell-mediated (ESC) gene transfer and Lentiviral Transfer of Oocytes and

Zygotes (Ahmad *et al.*2018). Transgenic dairy cows are able to produce casein content at higher level (Brophy et al., 2003) and are mastitis resistant Staphylococcus aureus (Wall et al., 2005). Introduction of new genes to the entirely different species is also possible through transgenesis (Magnus and Lali, 2008).

7.1 DNA Microinjection

In DNA microinjection the DNA is directly injected into the pronuclei of the embryos and was first documented technique. Due to the slow reproduction rate of the bovine animals and comparatively low embryo generation due to superovulation, the success through microinjection of DNA is only possible when DNA is microinjected to the blastocyst stage after in-vitro oocyte maturation and fertilisation followed by development till blastocyst stage (Krimpenfort *et al.* 1991).

7.2 Retrovirus-mediated gene transfer

Among the various gene transfer techniques, the retrovirus-mediated gene transfer was highly efficient (Nowrouzi et al. 2011). Retroviruses are used as vectors for gene transfer. These vectors efficiently transfer genes due to their affinity and infectivity for specific targeted cell and will result in the successful incorporation of transgene (Koo *et al.* 2014). The genetic material is transferred in the form of RNA into the host cells resulting in the chimera animal, these animals are then subjected to inbred for about 20 generations to produce homozygous transgenic animals which carries desired transgene in each and every cell of these offspring's (Manmohan and Niraj 2010).

7.3 Embryonic stem cell-mediated (ESC) gene transfer

This method involves the insertion of the target DNA into the embryonic stem cells that are cultured through *in-vitro* technique. The nature of the embryonic stem cell is pluripotent and are obtained from the inner cell mass of the blastocyst. The blastocyst stage of the embryo has the potential to differentiate into somatic cells, which lead to creation of complete organism. ESC allows the insertion, removal or modification of DNA sequences.

7.4 Lentivirus transfer of oocyte and embryo

The major drawback of Retrovirus-mediated gene transfer is that the silencing of transgenic locus started during embryonic development or shortly after birth (Chan *et al.* 1998). To overcome this drawback, the vector derived from Lentiviruses are used, the microinjection containing lentiviral vector is injected into the oocyte free from cumulus cells. The further study reported that the oocyte injected with the lentiviral vector produced efficient transgenic cattle and all these animals expressed enhanced green fluorescent protein (eGFP) expression (Hofmann *et al.* 2004). The same study reported that lentiviral gene can be either injected directly through sub zonal injection or indirectly through transduced nuclei into enucleated oocyte. The use of these vectors has some limitations such as integrated vectors sometimes show positional effect, the size of the vector genome restricted to only 8-10 kb (Wu et al. 2010), disruption of the endogenous gene through insertion mutagenesis by insertion of vector (Li and Lu 2010; Bednarczyk 2016).

Conclusion

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Innovative reproductive technologies have contributed enormously in the field of livestock industry. The implementation of AI technology not only maximizes animals' productivity but also provides opportunity to the individual sires with traits of superior quality for breeding and also reduces the risks of spreading sexually transmitted diseases. With the inclusion of semen sorting technology, selection of offspring of desired sex to increase profit in livestock industry has been made practical. The use of cryopreservation technology made possible the preservation of genetic material, semen or embryo for future use, thereby providing an effective method for the conservation of indigenous livestock, global genetic transport, gene banking, breeding line restoration, and for genetic rescue of endangered species. MOET and IVEP technology help to accelerate the transmission of desirable traits/genetic improvement by increasing offspring of selected males and females and the reduction of the generation interval in livestock populations in a shorter period of time compared to classical approaches. Moreover, with the development of disease-resistant animals and other approaches for enhancing animal production capacity, animal transgenesis has the potential to replace traditional.

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