STUDY ON CRYOPRESERVATION OF EMBRYOS BY USING SLOW FREEZING AND VITRIFICATION METHODS IN SAHIWAL COWS

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CERTIFICATE

This is to certify that the thesis entitled **"*STUDY ON CRYOPRESERVATION OF EMBRYOS BY USING SLOW FREEZING AND VITRIFICATION METHODS IN SAHIWAL COWS*"** submitted by **Ms. NIDHISHREE J JAKKALI,** I.D. No**. MVNK 1805** in partial fulfilment of the requirements for the award of degree of **MASTER OF VETERIARY SCIENCE** in **VETERINARY GYNAECOLOGY AND OBSTETRICS** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work carried out by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, association ship, fellowship or other similar titles.

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Affectionately Dedicated to
My Parents
and
My Family

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
ART	Assisted Reproductive Technology
B-mode	Brightness mode
BL	Blastocyst
Bo-IVC	Bovine -*in vitro* culture
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
BHV-I	Bovine herpes virus- I
BG	Butylene glycol
CO_2	Carbon dioxide
CL	Corpus Luteum
Cm	Centimetre
CA	California
CPs	Cryoprotectants
CPAs	Cryoprotectants
COC	Cumulus oocyte complex
CIDR	Controlled Internal Drug Releasing Device
°	Degree
°C	Degree Celsius
°C/min	Degree Celsius per Minute
d	Days
DCP	Digestible Crude Protein
Dex	Dextran
DMSO	Dimethyl sulfoxide
DT	Direct transfer
DFR	Dominant follicle removal
D1, D2 & D3	Devitrification media

E	East
Ecg	Equine chorionic gonadotropin
ET	Embryo Transfer
ET & IVF	Embryo transfer and *Invitro* fertilization
ETB	EmbryoTrans Biotech
EG	Ethylene glycol
et al.,	And other associates
Fig.	Figure
Fic	Ficoll
FSH	Follicle stimulating hormone
F/T	Frozen thawed
FTET	Fixed timed embryo transfer
G	Gravitational force
Gly	Glycerol
HBL	Hatching blastocysts
hrs	Hours
HDT & EG	Freezing media
IM	Intramuscular
I/V	Intravenous
IVC	*in vitro* culture
IVEP	*In vitro* Embryo Production
IVP	*In vitro* Production
IVFs	*In vitro* Fertilizations
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IETS	International Embryo Technology Society
ICM	Inner cell mass
IVC-ET	Embryo transfer medium
Kg	Kilogram

LN_2	Liquid Nitrogen
LH	Luteinizing hormone
µm	Micrometre
µl	Microlitre
M	Molar
Min.	Minutes
MHz	Mega Hertz
mm	Millimetre
mmHg	Millimetre of mercury
ml	Millilitre
Met	Methanol
mg	Milligram
MEM	Minimum Essential Media
mol/L	Moles per litre
MOET	Multiple ovulation and embryo transfer
n	Number
N	North
N_2	Nitrogen
O_2	Oxygen
OPS	Open pulled straw
OPU	Ovum Pickup
OPUs	Ovum Pickups
OPU – IVEP	Ovum Pickup – *In vitro* Embryo Production
pH	Potential of hydrogen
PVP	Polyvinyl pyrrolidone
PEG	Polyethylene glycol
PrOH	Propanediol
PG	Propylene glycol
%	Percentage

RGM	Rastriya Gokul Mission
Sec	Seconds
SC	Subcutaneous
SOF	Synthetic Oviductal Fluid
SOFaa	Synthetic Oviductal Fluid amino acid
TDN	Total Digestible Nutrients
TET	Timed Embryo Transfer
TAI	Timed Artificial Insemination
TALP	Tyrode’s Albumin Lactate Pyruvate
TCM-199	Tissue Culture Media- 199
TE	Trophoectoderm
THI	Temperature Humidity Index
UK	United Kingdom
USA	United States of America
USG	Ultrasonography
UPS	Uninterrupted power supply
V/W	Vitrified warmed
V1 & V2	Vitrification media
vs.	Versus
viz.	Namely or as follows
i.e.,	That is
–	Dash or minus
/	Or / Dividing slash
±	Plus or minus
<	Less than
>	More than
≥	More than or equal to

Introduction

I. INTRODUCTION

India is one of the mega-biodiversity countries in the world and is a home of about 11.54 percent of the total livestock population in the world. India has a total livestock population of 535.78 million out of which, 192.49 million are cattle (Livestock census, 2019). India has 37 registered pure indigenous cattle breeds including Sahiwal, Gir, Red Sindhi, Tharparkar (Swaroop, 2020). The Sahiwal originated in the dry Punjab region which lies along the Indian-Pakistani border and Sriganganagar districts of Rajasthan and due to its unique triats like tick-resistant, heat-tolerant, high milk production, high resistance to parasites (both internal and external), it is known as one of the best dairy breeds in India and Pakistan. Out of total 192.49 million cattle population, indigenous and nondescript cattle population is estimated as 139.82 million, which shows a decline of 6% in the total Indigenous (both descript and non-descript) cattle population over the previous census (Livestock census, 2019). This decline emphasizes the need for developing long term strategies for its conservation. Ovum Pickup – *In vitro* Embryo Production (OPU – IVEP) in combination with embryo cryopreservation and Embryo Transfer (ET) can be a viable alternative to conserve breeds, increase milk production and at the same time ensure faster multiplication of superior germplasm.

Ovum PickUp has virtually no drawbacks for the donor and can even have a therapeutic effect in some infertile donors affected by ovarian cystic condition or similar pathologies that compromise reproductive function. In this method, female starting from 6 months of age up to the third month of pregnancy and also soon after calving (2–3 weeks), is a suitable donor (Galli *et al.*, 2004). Ovum PickUp does not interfere with the normal

reproduction and production cycles of the donor. It can be performed sporadically or on a regular basis such as two times a week for many weeks or months (Galli *et al.*, 2004), which means that the number of calves that can be obtained per unit of time can be four to five times as large as with conventional ET (Gordon, 2004). The application of Ovum Pick-up technology, together with multistep embryo production *in vitro* (IVEP), represents a valid procedure for the recovery of oocytes from live donors and obtaining a large number of embryos (Galli *et al*., 2001). There is a growing interest in Bos indicus dairy breeds, in tropical areas due to their ability to adapt and produce milk under hot weather conditions. In this context, *in vitro* embryo production (IVEP) represents an interesting choice. Compared with *in vivo* embryo production, IVEP requires fewer viable sperm cells (Pontes *et al.,* 2010). Despite the advantages offered by IVEP, the major challenge to its greater dissemination is to improve embryonic survival after cryopreservation.

Embryo cryopreservation process is the most challenging aspect of embryo biotechnology, and despite advances in recent years, the results are still inconsistent (Sudano *et al*., 2013). Cryopreservation protocols are based on two variables: type and concentration of cryoprotectant and cooling rates (Vajta and Kuwayama, 2006). Currently, slow freezing (classic) and vitrification (ultra-rapid) are the two main methods used commercially for IVEP embryo cryopreservation (Saragusty and Arav, 2011). Cryopreservation methods aim to avoid the formation of intracellular ice crystals and to decrease the toxic effects generated by the cryoprotectant agent, minimizing the osmotic stress to the cells (Pryor *et al*., 2009).

In the classical slow-freezing protocol, the cooling rate is controlled to maintain a constant curve until the straws with embryos are immersed in the liquid nitrogen. The use of low concentrations of cryoprotectants is the main advantage of this technique since high concentrations are toxic to embryos. In addition, thawing process and the direct transfer (DT) of embryos to cows make the slow freezing protocol more efficient for commercial use. However, there can be ice crystal formation and damage to the structure of the embryo's membranes and organelles (Dode *et al*., 2013). In this way, the success of slow freezing and direct transfer of *in vitro* produced embryos invariably depends on the equilibrium between the rate of dehydration of the cell and the rate at which water is transformed into ice crystals (Visintin *et al*., 2002).

Vitrification is the predominant technique used for IVEP (Dode *et al*., 2013) due to its simple, fast and low-cost method (Sanches *et al*., 2016). In this method, a high-osmolarity solution is used so that the embryonic intracellular water exits rapidly, dehydrating the embryonic cells and making them permeable to the cryoprotectant. Thus, the embryo is able to withstand direct immersion in liquid nitrogen (-196ºC) without the formation of ice crystals (Vajta *et al*., 1998). On the other hand, high cryoprotectant concentrations have been described as promoting high cellular toxicity, even if exposed for a short period and a minimum volume of this solution (Vajta *et al*., 1998). Thus, different strategies have been developed for embryos to have rapid contact with liquid nitrogen and to reduce the volume of the cryoprotectant agent, such as the open pulled straw (OPS) (Vajta *et al*., 1998), cryoloop (Lane *et al*., 1999), microdroplets (Papis *et al*., 2000) and cryotop techniques (Kuwayama *et al*., 2005).

The total embryo production can sometimes be higher than the number of embryos to be transferred, so investment in research is necessary to develop an efficient protocol for the cryopreservation of the remaining embryos in a program (Sanches *et al.,* 2016). Therefore the present study was aimed to study and standardize the embryo cryopreservation methods of Sahiwal cow embryos. Two embryo cryopreservation methods were applied on embryos produced from Sahiwal cows after IVEP with the following objectives-

- To study cryopreservation of embryos by using slow freezing and vitrification methods.
- To compare the conception rate with fresh, slow freezed and vitrified embryos.

Review of Literature

II. REVIEW OF LITERATURE

2.1 Transvaginal Ultrasound- guided Ovum Pick-Up (OPU)

Pieterse *et al*. (1988) reported the first ultrasound-guided transvaginal ovum pick up (OPU) in the cow. Previously in IVEP technology all oocytes were collected from slaughtered donors but in recent years the Ovum Pick Up technique (OPU) has become a routine procedure (Galli *et al*., 2001) and is the biotechnological alternatives currently available to support breeding programs in this species (Galli *et al*., 2014). The major limitation with oocytes collection from slaughtered donors was lack of information on reproductive health of the animal and inability or impossibility to repeatedly collect oocytes especially from a genetically superior donor (Bols *et al*., 1995), hence researchers have moved towards different techniques of retrieval of oocytes from living animals, which includes ultrasound guided transvaginal OPU, laparoscopic OPU and OPU by laprotomy method. Among these methods, ultrasound guided transvaginal OPU is the best method, as this procedure is less traumatic to the ovary, vagina and especially the fornix, could be performed successfully in any stages of estrus cycle of donor, less risk of infection and higher degree of repeatability (Santl *et al*., 1998).

This technological advance has considerably widened the potential application of IVEP in genetic improvement of cattle by greatly increasing the number of offspring that are genetically superior. This method consists of the transvaginal aspiration of follicular oocytes with the aid of a scanner with an endovaginal probe. A guided needle secured to the probe is connected to a test tube and to a vacuum pump to aspirate the follicular fluid

and the oocyte contained in it. The donor is confined in a crush and given an epidural anaesthesia just before collection (Galli *et al*., 2004).

OPU has virtually no drawbacks for the donor and can even have a therapeutic effect in some infertile donors affected by ovarian cystic syndrome or similar pathologies that compromise reproductive function. Virtually any female starting from 6 months of age up to the third month of pregnancy and also soon after calving (2–3 weeks), is a suitable donor (Galli *et al.*, 2004). OPU does not interfere with the normal reproduction and production cycles of the donor. Oocytes can be recovered from normal or hormonally stimulated cows (Pieterse *et al*., 1988; Blondin *et al*., 2002; Chaubal *et al*., 2006 and 2007; Tribulo *et al*., 2011; da Silva *et al*., 2017 and Cavalieri *et al*., 2017). It can be performed sporadically or on a regular basis such as two times a week for many weeks or months (Galli *et al.*, 2004), which means that the number of calves that can be obtained per unit of time can be four to five times as large as with conventional ET (Gordon, 2004).

Ultrasound - guided transvaginal OPU has proved to be a reliable, repeatable and efficient technique for obtaining developmentally competent oocytes from live animals viz., cattle (Pieterse *et al*., 1988; Chaubal *et al*., 2006; Li *et al*., 2007; Pontes *et al*., 2009; Nagai *et al*., 2015; Monteiro *et al*., 2016 and Cavalieri *et al*., 2017).

Bousquet *et al.* (1999) demonstrated that a combination of mild superovulatory treatment with FSH and OPU can increase the number of embryos produced in a given time, therefore representing a valid alternative to conventional superovulation.

Blondin *et al*. (2002) showed that inducing follicular growth to simply increase the number of medium- and large-sized follicles is not sufficient to produce maximal numbers of competent COC. Induction of a preovulatory-type follicular environment is necessary to trigger COC to complete their cytoplasmic maturation so that oocytes are developmentally competent even before maturing them *in vitro*. For this, 48hrs coasting period plus LH administration, in association with a standard FSH superstimulation protocol, is sufficient to create these optimal *in vivo* follicular conditions.

Chaubal *et al*. (2006) concluded from his research work that DFR (dominant follicle removal) followed by 200 mg FSH administration (IM and SC) and subsequent OPU was the best protocol on a per cow per session basis for follicular response, oocyte recovery and embryo production. Cows were successfully stimulated repeatedly for 10 consecutive weeks, using a single weekly injection of a reduced dose of FSH [compared to the standard full dose used in routine multiple ovulation and embryo transfer (MOET)]. Besides avoiding ovarian hyperstimulation, this treatment apparently also reduced the stress associated with multiple FSH injections. This protocol produced the maximum number of embryos with optimal use of resources (in terms of FSH dose, number of OPUs/week and number of IVFs/week), which could be appealing in a commercial operation.

Chaubal *et al*. (2007) showed that administration of LH I/V 6 hrs prior to OPU without the use of CIDR during ovarian stimulation increased the oocytes of higher morphological grades and resulted in improved blastocyst development rate with 2.89 ± 0.4 blastocysts per cow per OPU session as compared to 1.56 ± 0.4, 1.56 ± 0.4 and 1.33 ± 0.4 for all other groups. Also concluded that, compared to single administration, multiple

FSH administration increased the available follicles for aspiration. He also concluded that the weekly ovarian stimulation (to maximize embryo harvest over a short interval), coupled with LH administration, though successful, but resulted in ovarian hypertrophy through follicular luteinization. This warrants a judicious use of these protocols, with adequate interval between successive rounds of stimulation and OPU when intended to be used over a longer time period.

Sendag *et al.* (2008) concluded that average follicle number in ovaries was higher in FSH group than eCG group. Oocyte yields per animal did not differ between FSH and eCG groups but the proportion of grade A oocytes was higher in the FSH group than in the eCG group. Likewise, rate of grade C oocytes in FSH group were lower than eCG group suggesting that ovarian response, follicle number in ovaries and oocyte quality are affected by the type of gonadotropin and FSH is better alternative than eCG for OPU treatment.

Cavalieri *et al*. (2017) revealed that donors that received the synchronization protocol pre-OPU showed a positive effects on *in vitro* embryo production in terms of greater number of embryos (5.9 ± 0.5 *vs.* 4.5 ± 0.4), higher rate of embryo production (45.8% *vs.* 38.5%) and higher mean number of conceptions per group (2.2 ± 0.2 *vs.* 1.6 ± 0.2) in relation to the group that did not receive hormonal treatment.

2.2 (*Invitro* Embryo Production) IVEP

The application of Ovum Pick-up (OPU) technology, together with multistep embryo production *in vitro* (IVEP), represents a valid procedure for the recovery of oocytes from live donors and the attainment of a large number of embryos, suitable for transfer or cryopreservation (Galli *et al*., 2001). There is a growing interest in Bos indicus dairy

breeds, in tropical areas due to their ability to adapt and produce milk under hot weather conditions. In this context, *in vitro* embryo production (IVEP) represents an interesting choice. Compared with *in vivo* production, IVEP requires fewer viable sperm cells (Pontes *et al.* 2010).

IVEP involves three steps, oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) (Gordon, 2004). In IVM, good quality oocytes are selected and kept in maturation media for 20-24 hrs in CO_2 incubator at 38.5°C, 5% CO_2 and 90-95% relative humidity. After completion of IVM, oocytes are co-incubated with spermatozoa for up to 16 to 18 hrs while undergoing IVF (Gordon, 2004). After IVF, the fertilized oocytes are submitted to IVC media for 6-7 days until they reach the blastocyst stage. In general, 20% to 40% of the cultured presumptive zygotes will reach the blastocyst stage (Rizos *et al*., 2008). After reaching the blastocyst stage, embryo transfer is performed following similar procedures as with *in vivo* blastocysts, or embryos are cryopreserved.

For IVEP, different medias are used which have necessary elements for the development and maintenance of the oocytes, sperms and embryos. Multiple sources are added to medias like; balanced salt solution, protein, hormones, antioxidants, antibiotics, growth factors and sugar, to create an optimal environment for the oocytes and sperms and subsequently to obtain high quality of embryos (Pawshe *et al.,* 1996; Rao *et al*., 2002). Several media, including; Tissue Culture Media- 199 (TCM-199) (Kharche *et al.,* 2006; Amer *et al*., 2008), Tyrode's Albumin Lactate Pyruvate (TALP) stocks (Parrish *et al*., 1986), Synthetic Oviductal Fluid (SOF) (Gandhi *et al*., 2000), and Minimum Essential

Media (MEM) (Mohammadi *et al.,* 2006) have been used for oocytes maturation, fertilization and embryo development in mammals.

Many significant additions are made to the growing media to contribute oocyte maturation and fertilization in an environment similar to the living body. These additions help in oocyte development because they contain essential materials for the process of oocyte maturation (Chiamenti *et al*., 2010). The IVF culture also should be useful in providing sperm the needed movement and adaptation, which eventually leads to its union with the ova and then the beginning of embryonic development (Mahoete, 2010). With the increasing implementation of IVEP of bovine embryos worldwide for commercial use, there is an increased focus on optimizing the yield of blastocysts. For this reason, in recent years, many companies have come up with commercially available medias having all essential components for oocyte, sperm and embryos development. These helps in performing all the procedures easily and with less contamination, in turn which helps in giving better results.

In human as well as in bovine IVF the monoculture medium system is gaining popularity. The monoculture medium is supplemented with all the required compounds to sustain embryo development to the blastocyst stage, and is based on letting the embryo choose the nutrients and components needed for an optimum development during the entire culture period (Gardner and Lane, 2002). It has been suggested that monoculture medium system is as efficient as the sequential medium system (Macklon *et al*., 2002). Knowing that the embryos worst enemy is the fluctuations, in particularly, of pH and temperature

(Swain, 2010), a monoculture medium system has the advantage of decreasing the number of manipulations and the length of time the embryo is out of the incubator.

Refined serum-free culture conditions, based on BSA supplementation, have been developed allowing for improved fetal development and calving (George *et al*., 2008), and in 2013 an entire serum-free ready-to-use media suite for all the steps, maturation, fertilization and culture, was made commercially available by IVF Bioscience, UK, combining synthetic serum replacements and BSA (Hyttel *et al*., 2019).

Nielsen *et al.* (2015) concluded that the developmental rates and gene expression of *in vitro*-produced bovine blastocysts were affected by the use of different culture media. Increased blastocyst rates, apparently superior embryo quality, and more abundant gene expression were achieved when blastocysts were cultured in Bo-IVC culture media (IVF Biosciences) compared with SOF.

Pryor *et al.* (2016) showed from his research that ETB (EmbryoTrans Biotech) media was superior to control media for percent viable, HBL (hatching blastocysts), and combined HBL/expanded BL (51.9, 23.9, 45.8% *vs.* 29.2, 5.8, 20.5, respectively). Also concluded that ETB media produced more high-quality embryos than control media under varying conditions experienced by commercial IVF companies.

2.3 Cryopreservation of embryos

Cryopreservation is a technology for the long term preservation of mammalian cells, gametes and zygotes or embryos at -196° C in LN_2, while keeping the cells or tissue "alive". Cryopreservation and storage of the embryos produced *in vivo* or *in vitro* is a

crucial step of commercial application of the embryo transfer, which allows for efficient utilization of recipients, reduces the need to move cattle, allows an efficient means for marketing of genetics, and greatly facilitates the international movement of bovine genetics. In domestic animals, it helps in breeding and reproduction. It also provides methodology to maintain genetic diversity, conservation and preservation of endangered species.

Embryo cryopreservation process is the most challenging aspect of embryo biotechnology, and despite advances in recent years, the results are still inconsistent (Sudano *et al*., 2013). Cryopreservation protocols are based on two variables: type and concentration of cryoprotectant and cooling rates (Vajta and Kuwayama, 2006). Currently, slow freezing (classic) and vitrification (ultra-rapid) are the two main methods used commercially for IVEP embryo cryopreservation (Saragusty and Arav, 2011).

Mammalian cells are stored at -196ºC, the temperature of LN_2 where there is no active cellular metabolism. The critical steps of cryopreservation in terms of cellular damage are, cooling to -196°C and the subsequent thawing to 37°C. This is because water in the cell solidifies in the form of ice crystals when it is cooled to below its freezing point. Since ice is less dense than liquid water, the ice crystals occupy a greater volume than the liquid water from which they were formed. This volume expansion causes increased pressure and damage to intracellular organelles. As water transitions takes place from liquid to ice, any solutes in the liquid phase are excluded from the solid. For avoidance of ice crystals formation therefore is one of the goals of successful cryopreservation, which requires cryoprotectants.

As the temperature drops and the solid form proliferates, the concentration of electrolytes and other solutes in the remaining unfrozen solution rises dramatically, resulting in lowering of the freezing temperature of the solution (Lovelock, 1954). However, these high solutes concentrations can be quite toxic to intracellular proteins and other cellular components in the form of osmotic injury. Thus the aim of cryopreservation is to avoid both ice crystals formation and the rise in intracellular osmolarity.

Similarly, during thawing the solid ice melts and releases free water, resulting in decreasing osmolarity of the cellular solution. When re-warming is too rapid, a sudden drop in the extracellular osmotic pressure may lead to a rapid shift of free water into the cell, leading to swelling and cellular damage. On the other hand, when the re-warming process is slow, there is a danger of free water re-crystallizing, thus causing further cellular damage (Mazur, 1980). This phenomenon is sometimes referred to as osmotic shock and its avoidance is a third major goal of a successful cryopreservation.

Thus, cryopreservation methods aim to avoid the formation of intracellular ice crystals, to decrease the toxic effects generated by the cryoprotectant agent and minimizing the osmotic stress/shock to the cells at both high and low temperatures (Pryor *et al*., 2009).

2.3.1 Cryoprotectants

Cryoprotectant is any substance that aids in cell survival during freezing and thawing (Bondioli, 2014). Cryoprotectants should be non- or minimally toxic, able to penetrate cell membranes easily, and able to bind either with electrolytes (to increase concentration in the freezing process) or with water molecules (to delay freezing). What basically cryoprotectants do is after dissolving in preserving solution it lowers the melting

point of the solution so that tissue or cells can freeze within its surroundings but not within its cells. Cryoprotectant compounds can be divided into two general classes- **Permeating** and **Non- Permeating** cryoprotectant.

2.3.1.1 Permeating cryoprotectant or Intracellular CPAs

Permeating cryoprotectants have ability to penetrate cell membranes, achieve adequate concentrations in the cytoplasm and act intra-cellularly. These compounds have a small molecular weight and a polar molecular structure that can mimic that of water. Their hydrogen bonds with water molecules prevent ice crystallization by lowering the freezing point. These chemical compounds can generating an osmotic gradient, diffuses through cellular membranes of oocytes and embryos, permeate the cell, and equilibrate within the cytoplasm, replacing the intracellular water without over-dehydrating the cell; penetrated CPAs solidify at lower temperatures than water and thus subsequently reduce the amount of intracellular ice formation at that given temperature (Chian and Quinn, 2010). There is a fact that CPAs permeate the cell membrane slower than water, the loss of intracellular water is faster than the gain of CPAs inside the cell, which causes a change in cell volume over time, producing a typical shrink–swell curve (De Santis and Coticchio, 2011). Commonly used permeating CPAs are: BG:- butylene glycol, PrOH:- propanediol, PG:- propylene glycol, DMSO:- dimethyl sulfoxide, Gly:- glycerol, EG:- ethylene glycol and Met:- methanol.

At adequate concentrations they inhibit the formation of ice crystals and lead to the development of a solid or glasslike state. This is the so-called vitrified state in which water solidifies without crystals formation or expansion. However, the permeating

cryoprotectants may also have a toxic effect at high enough concentrations. This requires that the cell be exposed to this solution either for a very short period of time, if the cryoprotectant is used at room temperature (vitrification techniques) or at very low temperatures, in which the metabolic rate of the cell is very low (slow-freezing protocols). The permeating cryoprotectants also protect the cell from the solution effects, as free water solidifies immediately to ice, the remaining solution will contain progressively higher concentrations of both cryoprotectant and electrolytes.

2.3.1.2 Non-permeating cryoprotectant or Extracellular CPAs

Non-permeating cryoprotectants remain in the extracellular space, increases its osmolarity. It acts by drawing free water from within the cell, thus dehydrating the intracellular space and reduces intracellular ice-crystal formation. Besides, the extracellular CPAs are often included in media for warming or thawing of cells to help to prevent traumatic cell expansion during the process of rehydration. These extracellular CPA compounds are usually high-molecular-weight polymers (Wowk, 2007), which do not pass through the membrane of the cells, stabilize cell membranes during the post-thaw phase, and increase the viscosity of the solution. Commonly used non-permeating CPAs are- Glucose, Sucrose, Trehalose, Ficoll, PVP: polyvinyl pyrrolidone, Fic: Ficoll, Dex: dextran and PEG: polyethylene glycol.

When they are used in combination with a permeating cryoprotectant, the net concentration of the permeating cryoprotectant is increased in the intracellular space. This facilitates the permeating cryoprotectant action in preventing ice-crystals formation. Furthermore, during thawing, the water generated by the melting ice rapidly decreases the

extracellular osmotic pressure. Osmotic shock may occur if the intracellular cryoprotectant cannot diffuse out quickly enough to prevent excessive influx of free water and the swelling, or even rupture of the cell. Therefore, freezing and thawing protocols commonly use a high concentration of non-permeating cryoprotectant during the thawing phase.

2.3.1.3 Combination of CPAs

Most cryopreservation solutions used for oocytes and embryos are made up in a physiological solution, adding intracellular CPAs (PrOH, DMSO, EG, Gly) and extracellular CPAs (sucrose, trehalose) with a protein which improves handling characteristics and membrane stability and can reduce toxicity. The conditions under which oocytes or embryos are exposed to CPA are very important, knowing that cell membrane permeability increases at higher temperature; when CPAs are loaded at 37 °C, the rate of exchange of water and CPA between the intra- and extracellular spaces increases and the shrink–swell contraction response is reduced in comparison to dehydration carried out at room temperature (Paynter *et al*., 2001).

2.3.2 Cryoinjuries

For cells to be cryopreserved without a decrease in survival for the duration of storage, they are preserved in liquid nitrogen (−196°C), which is used for maintaining the temperature. Not only during cooling but also during processing before cooling, during warming, and at recovery, cells are at risk of various types of injury. To minimize the decrease in survival, these injuries must be minimized.

2.3.2.1 Chilling injury

In vitro-derived embryos are more sensitive to cryopreservation than *in vivo* fertilized embryos. Certain types of oocytes/different stages of embryos (e.g. pig oocytes, pig embryos before the peri hatching stage, bovine oocytes and bovine embryos at early cleavage stages) are sensitive to cooling to below +20°C (Leibo *et al*., 1996). Cells, having chilling sensitivity, appear dark with cytoplasmic lipid droplets, which are thought to be associated with the chilling injury. Therefore, the successful cryopreservation of pig embryos has been achieved by using embryos at the peri hatching stage when there are fewer droplets or by removing the droplets (Esaki *et al*., 2004).

2.3.2.2 Intracellular ice formation

Mammalian oocytes/embryos are large cell masses, a large proportion of which is occupied by water. When they are cooled to low temperatures, intracellular ice crystals are liable to form. This is fatal because even a small amount of ice is likely to become larger and destroy the cellular structure. To prevent this, cells must be concentrated so that vitrification occurs below the glass transition temperature. Vitrification is the solidification of a solution without crystallization. For intracellular vitrification in mammalian oocytes and embryos, inclusion of a cryoprotectant is essential. Cryoprotectants are small neutral solutes, such as glycerol, ethylene glycol, dimethylsulfoxide (DMSO), and propylene glycol, which can permeate into the cell. In slow freezing, cells are concentrated gradually during slow cooling, whereas in vitrification, cells are concentrated upon direct suspension in a concentrated solution.

2.3.2.3 Extracellular ice

If mammalian embryos are suspended in a physiological solution and cooled to low temperatures, intracellular ice forms and the embryos will die. If the sample is seeded and cooled slowly, the embryos may be crushed by the extracellular ice, as the unfrozen fraction in which the cells are located becomes smaller because the cells are large (Schneider and Mazur, 1987). Extracellular ice can cause another type of injury. As the amount of extracellular water in the solution is reduced by the conversion to ice, the solution becomes concentrated, exposing the cells to high concentrations of solutes (i.e. electrolytes). However, by including a cryoprotectant in the solution, these injuries can be prevented.

2.3.2.4 Toxicity of cryoprotectants

For the cryopreservation of embryos, the inclusion of a cryoprotectant that can permeate into the cell is essential. The mechanism of the protective action of permeating agents is considered the same, but their toxicities are different. For slow freezing, the concentration is limited to 1–2 mol/L, and the toxicity is relatively low. In vitrification, however, the concentration can be as high as 8 mol/L, and the selection of a low toxicity agent is more important. In addition to permeating agents, small saccharides and macromolecules are frequently added to the solution (Kasai, 1997). However, the toxicity of non-permeating agents is quite low.

2.3.2.5 Fracture damage

When cryopreserved embryos are recovered, they are occasionally found to be cracked. This physical injury, called fracture damage, is thought to be caused by non uniform changes in the volume of the liquid and solid phases of the medium during rapid

phase changes. Fracture damage can be reduced by reducing the cooling and warming velocities during passage through the temperature range where the phase change would occur (approximately −130°C) (Rall and Meyer, 1989). Fracture damage is also related to the container used for the storage of embryos; plastic mini-straws result in less damage (Van den Abbeel and Van Steirteghem, 2000). In vitrification using straws, fracture damage can be prevented completely by cooling and warming to the phase transition temperature in a gas phase (Kasai *et al*., 1996).

2.3.2.6 Osmotic swelling

Just after warming, cryopreserved embryos contain a permeated cryoprotectant, which has to be removed. If the cells are directly recovered in an isotonic solution, they risk injury from osmotic swelling, because water diffuses in far more rapidly than the cryoprotectant diffuses out. The sensitivity of oocytes/ embryos to osmotic swelling differs with the stage of development. But at all stages, cryopreserved cells just after warming are more sensitive than fresh ones (Pedro *et al*., 1997b and Edashige *et al*., 1999). The most common strategy for preventing this injury is to dilute embryos with a hypertonic solution containing sucrose as a non-permeating solute to counteract the inflow of excess water (Kasai *et al*., 1980). Inclusion of sucrose in the cryopreservation solution may also help prevent cells from swelling, as small saccharides such as sucrose promote cell shrinkage before dilution (Kasai *et al*., 1990).

2.3.2.7 Osmotic shrinkage

Upon removal of the permeated cryoprotectant out of the cells by diffusion using a sucrose solution, embryos remain shrunken. This hypertonic shrinkage may also be

injurious to the cells. As in the case of hypotonic stress, cryopreserved cells just after warming are sensitive to this injury (Pedro *et al*., 1997a). Therefore, after diffusion of the permeated cryoprotectant out of the cell in a hypertonic solution, the embryos should be transferred into a less hypertonic solution, and finally into an isotonic solution.

2.3.3 Selection of embryos for cryopreservation

Embryo evaluation is one of the most critical steps of the embryo selection and transfer procedure. The IETS Manual states that embryos must be graded based on a 1 to 9 point system to determine the stage of development and a 1 to 4 system to determine embryo quality. Grade 1 embryos survive well to the freezing/thawing procedure and are recommended for international trade; whereas Grade 2 and 3 must be transferred fresh into suitable recipients (Bó and Mapletoft, 2013).

2.3.4 Slow freezing

In the classical slow-freezing protocol, the cooling rate is controlled to maintain a constant curve until the straws with embryos are immersed in the liquid nitrogen. Slow programmable freezing was initially the standard method of embryo cryopreservation in most ART centres world-wide. In this method, there is use of low concentrations of cryoprotectants, at temperatures in which there is still active cellular metabolism, which is the main advantage of this technique since high concentrations are toxic to embryos. The temperature then is lowered gradually (about 0.3– 0.5°C/min) to the seeding temperature at - 6°C. During this time, all of the solution remains liquid.

At a temperature of - 6°C, ice crystals may be induced in the solution by introducing a seed, a small crystal of ice that allows other water molecules to undergo crystallization. Seeding commonly is accomplished by touching the outside of the cryopreservation vessel (conventional straw) with a very cold instrument (pre-cooled forceps), which then induces a small ice crystal to form in the area at which the cryostraw is touched. Therefore, seeding is performed in an area distant from the embryo, so that the ice crystal grows gradually toward the embryo. After equilibration, the temperature is decreased gradually to a final temperature of - 32°C or - 35°C. During this time, the ice crystal gradually propagates in the extracellular medium, thus further increasing the concentration of cryoprotectants, particularly in the intracellular space, which is further dehydrated through the use of non permeating cryoprotectants. The very slow rate of cooling (0.33°C/min) allows gradual diffusion into the embryo of additional permeating cryoprotectant, while maintaining equilibrium with the extracellular space. At the final temperature of - 32°C or - 35°C, the metabolic rate of the embryo is quite slow, further limiting the toxicity of the increasing concentrations of the cryoprotectants. The freezing vessel now is plunged into LN_2, and the remaining non solidified solution is converted to a solid state at - 196°C.

During thawing, rapid transition of temperature is preferred to prevent re-crystallization of water with the potential for ice-crystal damage. In addition, thawing process and the direct transfer (DT) of embryos to cows make the slow freezing protocol more efficient for commercial use. However, there can be ice crystal formation and damage to the structure of the embryo’s membranes and organelles (Dode *et al*., 2013). In this way, the success of slow freezing and direct transfer of *in vitro* produced embryos invariably

depends on the equilibrium between the rate of dehydration of the cell and the rate at which water is transformed into ice crystals (Visintin *et al*., 2002).

2.3.5 Vitrification

Vitrification is a physical process by which the cells are frozen rapidly in a concentrated solution of cryoprotectants which solidifies during cooling without the formation of intracellular and extracellular ice crystals and represent a milestone in cryopreservation techniques. The solid, called glass, retains the normal molecular and ionic distributions of the liquid state and is therefore usually considered to be an extremely viscous, supercooled liquid. More recent studies have demonstrated that vitrification is an alternative to traditional slow freezing method for IVEP embryos (Dode *et al*., 2013), which avoids the chilling injury, ice crystal formation (Gupta *et al*., 2007), eliminates the use of a machine to control the cooling rate and is simple, fast and low-cost method (Sanches *et al*., 2016). During vitrification, permeating cryoprotectants are added at a high concentration, while the cell is still at room temperature and this high-osmolarity solution is used so that the embryonic intracellular water exits rapidly, dehydrating the embryonic cells and making them permeable to the cryoprotectant.

Thus, the embryo is able to withstand direct immersion in liquid nitrogen (-196ºC) without the formation of ice crystals (Vajta *et al*., 1998). On the other hand, high cryoprotectant concentrations have been described as promoting high cellular toxicity, (Vajta *et al*., 1998). Thus different methods have been used to reduce this ‘solution effect’: (i) short time of exposure to CPs (Takagi *et al*., 1993), (ii) use of low toxicity CPs (Rall *et al*., 1987) or mixtures of them, (iii) addition of nonpermeating CPs (Fahy *et al*., 1984)

which is very useful either because of shrinkage of the oocyte or embryos due to rapid exit of water from inside to outside, lowers the intracellular crystallization during rapid cooling and warming (Rall *et al*., 1987) or because of the reduction of the amount of the CP that penetrates the cell thus reducing the possible toxic effect, (iv) exposure at low temperatures (Rall *et al*., 1987), (v) reduction of the CP concentration (Rall *et al*., 1987) and (vi) reducing the volume of the cryoprotectant agent.

Volume of cryoprotectant can be reduced with different methods such as the open pulled straw (OPS; Vajta *et al*., 1998), cryoloop (Lane *et al*., 1999), microdroplets (Papis *et al*., 2000) and cryotop techniques (Kuwayama *et al*., 2005). But none of these are closed system and there are chances of potential contamination by liquid nitrogen such as hepatitis (Tedder *et al*., 1995), human immunodeficiency virus-I (Benifla *et al*., 2000), Bovine viral diarrhoea virus (BVDV) and bovine herpes virus- I (BHV-I) (Bielanski *et al*., 2000). Today, there are heat-sealed closed system devices available commercially to prevent this potential contamination; these devices include the CryoTip (Irvine Scientific, Irvine, CA) and the High Security Vitrification Kit (Cryo Bio System, L'Aigle, France).

2.3.6 Hatching and Conception rates with different cryopreservation methods

2.3.6.1 Fresh embryo transfer

Ambrose *et al.* (1999) reported that the cows that received IVP fresh embryos had a greater pregnancy rate at 45 to 52 d (14.3% ± 2.3%) than did cows that received a IVP frozen-thawed embryo (4.8% ± 2.3%) or timed insemination (4.9 ± 2.3%).

Al-Katanani *et al.* (2002) concluded that the pregnancy rates can be improved under heat stress condition with ET of fresh embryos. Also showed that the pregnancy rate on day 45 was higher with TET-Fresh (19.0 ± 5.0) than TAI (6.2 ± 3.6) and TET-Vitrified (6.5 ± 4.1%).

Moreira da Silva and Metelo (2005) showed that after 24 hrs of culture, embryo survival rate for slow-freezing/thawed (n = 23), vitrified/thawed (n = 20) and control embryos (n = 20) was 39, 27 and 90%, and 35, 14 and 65% after 48 hrs of culture, respectively.

Assumpção *et al*. (2008) showed that the *in vitro* survival rate (hatching at 96 hrs) of embryos was 68.4% for non cryopreserved embryos, 58.8% for embryos cryopreserved by slow-freezing method with cooling rate of 0.5°C/min and 36.9% for embryos Vitrified using Gly + EG.

Block *et al.* (2010) showed that the percentage of cows pregnant with fresh embryos were 56.3% (day 32), 52.6% (d 46), and 50.5% (d 76). And also concluded that with fresh embryos pregnancy rate was higher when compared with Timed AI cows or cows that received vitrified embryos.

Stewart *et al.* (2011) reported that the percentage of cow pregnancy at d 40 ± 7 of gestation was greater for the ET-Fresh (42.1%) when compared with the ET-Vitrified (29.3%) and AI (18.3%) groups among all cows.

Sanches *et al.* (2016) showed that the conception rates were 51.35±1.87% (133/259) for the fresh embryos, 35.89±3.87% (84/234) for the vitrified embryos and 40.19±4.65% (125/311) for the frozen directly transferred embryos.

Ismirandy *et al.* (2020) showed from their results that the recipient pregnancy rate of Bali cattle using fresh embryos (5/12, 41%) was higher compare to the frozen embryos (1/8, 12.5%).

2.3.6.2 Slow freezed embryos

Sommerfeld and Niemann (1999) reported that the controlled freezing (0.3°C/min to -35°C) of expanded day 7 blastocysts resulted in a hatching rate of 81%, which was similar to that of the non frozen embryos (76%) in 3.6 M EG and upon direct transfer of day 7 expanded blastocysts, frozen in 3.6 M EG, a pregnancy rate of 43% was achieved which was higher than the transfer of other developmental stages. Upon vitrification the hatching rates were 12% with IVP day 7 and day 8 blastocysts and 42% with expanded blastocysts in 7.2 M EG.

Kaidi *et al.* (2001) noticed that the hatching rates were 76 ± 7 % for fresh embryos, 49 ± 6.6 % for frozen embryos and 55 ± 5% for vitrified embryos.

Říha *et al.* (2002) reported that the pregnancy rates with IVP embryos, slow freezed and direct transfer were 72% (18/25) and with IVP vitrified embryos were 50% (12/24).

Guignot *et al.* (2006) showed that there was no significant difference between cryopreservation techniques: 69% versus 48% of kidding and 45% versus 35% of embryo

survival rates were obtained with slow freezing and standard vitrification of goat embryos, respectively.

Assumpção *et al*. (2008) suggested from the results that IVP bovine expanded blastocysts at days 7, 8 and 9 of IVC can be efficiently cryopreserved by slow freezing in EG and vitrification in Gly + EG in emcare® holding solution. The higher rates of embryo survival after thawing show that slow-freezing with 0.5°C/min was the best method to cryopreserve *in vitro*-produced bovine embryos with 58.8% hatching at 96 hrs when compared to Vitrification using Gly + EG yielded 36.9% hatching at 96 hrs.

Nicacio *et al.* (2012) stated that hatching rates were higher for slow freezing group embryos (44.65 ± 5.94% and 11.65 ± 3.37%) than vitrification groups (9.43 ± 6.77% and 8.67 ± 4.47%) when co-cultured in TCM199 and in SOFaa respectively.

Sanches *et al.* (2016) demonstrated that *in vitro* produced embryos with sexed semen could be directly transferred into recipient cows with similar conception rates to vitrified embryos. The conception rates in this study were 51.35 ± 1.87% (133/259) for fresh embryos, 40.19 ± 4.65% (125/311) for frozen embryos and 35.89 ± 3.87% (84/234) for vitrified embryos. They showed that the use of frozen embryos in direct transfer provides easier logistics and a more practical approach for the transfer of IVP embryos on dairy farms.

Fujii *et al.* (2017) showed that the rates of dead cells in *in-vivo* produced elongating conceptuses, cryopreserved by slow freezing were comparable to those in fresh elongating conceptuses. In addition, they obtained healthy calves from 2 recipients by the transfer of 3 elongating conceptuses cryopreserved by slow freezing.

Gómez *et al.* (2020) showed that 40 days pregnancy rates and birth rates with bovine embryos undergoing Frozen thawed (F/T) [22/40 (55%) and 18/40 (45%)], were similar to embryos transferred fresh [19/30 (63%) and 14/30 (47%)] and after Vitrified warmed (V/W) [29/47 (62%) and 25/47 (53%)].

2.3.6.3 Vitrified embryos

Agca *et al*. (1998) stated that the pregnancy rate of vitrified embryos 16/10 (63%) did not differ from that of fresh embryos 35/24 (68%). However, pregnancy rate of conventionally frozen embryos 17/7 (41%) was lower than that of fresh or vitrified embryos.

Lane *et al*., (1999) concluded that Cryoloop vitrification was able to cryopreserve mouse and human blastocysts without any reduction in the ability to re-expand and hatch in culture.

Nedambale *et al*., (2004) showed higher embryo development and hatching rates with vitrification at 6 hrs (71%), 24 hrs (64%), and 48 hrs (60%) post-warming compared to slow freezing (48, 40, and 31%, respectively). Following transfer of vitrified embryos to synchronized recipients, a 30% (3/10) pregnancy rate was obtained.

Manjunatha (2006) showed from his study that best hatching rate (22%) for buffalo morulae was achieved after vitrification with EG + DMSO exposure time of 2 minutes and the best hatching rate (46 %) for buffalo blastocyst was achieved after vitrification with EG + DMSO exposure time of 4 minutes.

Mucci *et al.* (2006) demonstrated higher Embryo survival (hatching rate) with vitrified embryos (43%) than slow-frozen embryos (12%).

Valojerdi *et al.* (2009) reported that vitrification is an efficient method in contrast to slow freezing for cryopreservation of human cleavage stage embryos. Also showed that the survival rate in the vitrification group versus slow freezing group was 96.9% vs. 82.8% respectively and the clinical pregnancy rate was 40.5% vs. 21.4% respectively.

Rodriguez *et al.* (2012) concluded that the re-expansion and hatching rates were higher for *in vitro*-produced vitrified embryos (155/223, 69% and 132/223, 59%) than *in vitro*-produced embryos cryopreserved by slow freezing (89/222, 40% and 45/222, 20%).

Caamaño *et al.* (2014) showed that vitrification is a better alternative than slow-rate freezing with ethylene glycol for cryopreserving IVP bovine blastocysts. IVP blastocysts produced in their experiment were either vitrified by (Cryologic Vitrification Method) CVM or subjected to slow freezing and they observed no differences between these cryopreservation techniques in terms of the survival and quality variables at 24 hrs. However, at 48 hrs they observed higher hatching rates (53.6±10.2) with vitrified embryos than with slow freezed embryos (32.5±10.2).

Gupta *et al.* (2016) reported different blastocyst rates among the treatments: unfrozen control (78 ± 3.6%), vitrification (52 ± 4.6%) and slow freezing (35 ± 4.2%) when morula were subjected to vitrification or slow freezing or no freezing. And concluded that vitrification is a more desirable technique than slow freezing for cryopreservation of IVP cattle morulae.

Do *et al.* (2017) demonstrated better post-recovery viability of *in vitro*-derived cattle embryos following vitrification than by slow freezing at both early and late stages of development. The hatching rate of expanded blastocysts after 48 hrs culture was significantly less for slow freezing group (31/47; 66.0%) than for both the short equilibration vitrification (46/51; 90.2%) and long equilibration vitrification groups (42/50; 84%).

Do *et al.* (2018) stated that the pregnancy rate of recipient female cattle into which *in vitro*-derived vitrified embryos (EG + DMSO) transferred were 40% (d35), 40% (d60) and 40% (d90) which was similar to the pregnancy rates of recipients into which *in vitro*-derived freshly processed embryos transferred were 41.3% (d35), 37.6% (d60) and 37.6% (d90).

Zarate-Guevara *et al.* (2018) concluded that the bovine blastocysts produced *in vivo* and cryopreserved by vitrification produce a higher gestation rate (64%) than those cryopreserved by slow conventional freezing (8%).

Gu *et al.* (2019) showed superiority on live birth rates and non-inferiority on safety perinatal outcomes from vitrified human ET, in comparison with slow-frozen human ET.

Gómez *et al.* (2020) showed that V/W bovine embryos improved survival over F/T bovine embryos (live and hatching rates at 2hrs, 24hrs and 48hrs). After F/T, embryos had lower cell counts in the (Inner cell mass) ICM, (Trophoectoderm) TE and total cells than after V/W.

Materials and Methods

III. MATERIALS AND METHODS

3.1 Experimental Location

The present study was undertaken at Livestock Farm Complex, College of Veterinary Science, Korutla, Jagitial district, Telangana (latitude: 18° 49′36.71”N; longitude: 78° 42′ 50.39”E; altitude: 295.99 m above mean sea level) under the ET & IVF Project (RGM), Dept. Of VGO, CVSc, Korutla during the period between October 2020 and November 2021. The climate in the region was semi-arid to sub humid with hot summer and cool winter. The decennial average rainfall was 922 mm and the ambient temperature range from a lowest of 13°C during January to a peak of about 45°C during May/June.

3.2 Experimental Animals

Sahiwal cows (*Bos indicus*) aged 3-6 years and weighing between 250 and 450 kg body weight were selected as oocyte donors through (Ovum Pick-Up) OPU.

The daily ration of each animal consists of 2-3 Kg high protein feed containing 17% DCP and 70% TDN, 8-12 kg chopped fodder and 3-5Kg paddy straw. Water was available continuously. Animals were maintained under hygienic and optimum management conditions in loose housing system with a large, open paddock for free movement. Health and vaccination protocols were followed as per standard schedule.

3.3 Ovum pick up

3.3.1 Equipment: The total setup of the OPU includes

1. Ultrasound machine (Exago, Nanuk, 930, IMV imaging, USA)
2. Transvaginal probe (Exago, Nanuk, 930, IMV imaging, USA)
3. Aspiration needle, needle guide and aspiration line carrier.
4. Aspiration line and 50ml centrifuge tube.
5. Vacuum pump along with dry block heater and foot operated pedal switch (TED Bomba de Aspiracao Touch., Brasil).

The ultrasound machine and the vacuum pump were placed on the right side of the animal chute and connected through an uninterrupted power supply (UPS) system to provide a uniform electric current. A real-time B mode ultrasound scanner (Exago, Nanuk 930, IMV imaging, USA) equipped with a multi frequency (5-9 MHz) probe was used for Ovum Pick-Up (Plate 1). The transvaginal probe (Plate 2) was equipped with a stainless steel needle and aspiration line carrier (56 cm long) and a needle guide (Plate 3). The aspiration needle (disposable hypodermic needle, 18-gauge, 82 mm in length) (Plate 3) was connected to a 50-mL conical centrifuge tube (Tarsons Spinwin Conical tubes, Kolkata) (Plate 4) via aspiration line which is passed through needle and aspiration line carrier (Plate 4). The aspiration line includes a silicon tubing system of 1.1mm inner diameter and 120 cm length. Vacuum pump (Plate 5) (TED Bomba de Aspiracao Touch., Brasil) equipped with dry block heater for placing conical tubes connected to wireless foot operated pedal switch (Plate 5) was used to create a vacuum of 70-75mmHg of continuous negative pressure, generating a fluid flow of 16-20 ml per minute. The conical centrifuge tube is placed in dry block heater to maintain constant temperature.

Plate 1: Ultrasound scanner fitted with transvaginal probe

Plate 2: Transvaginal probe

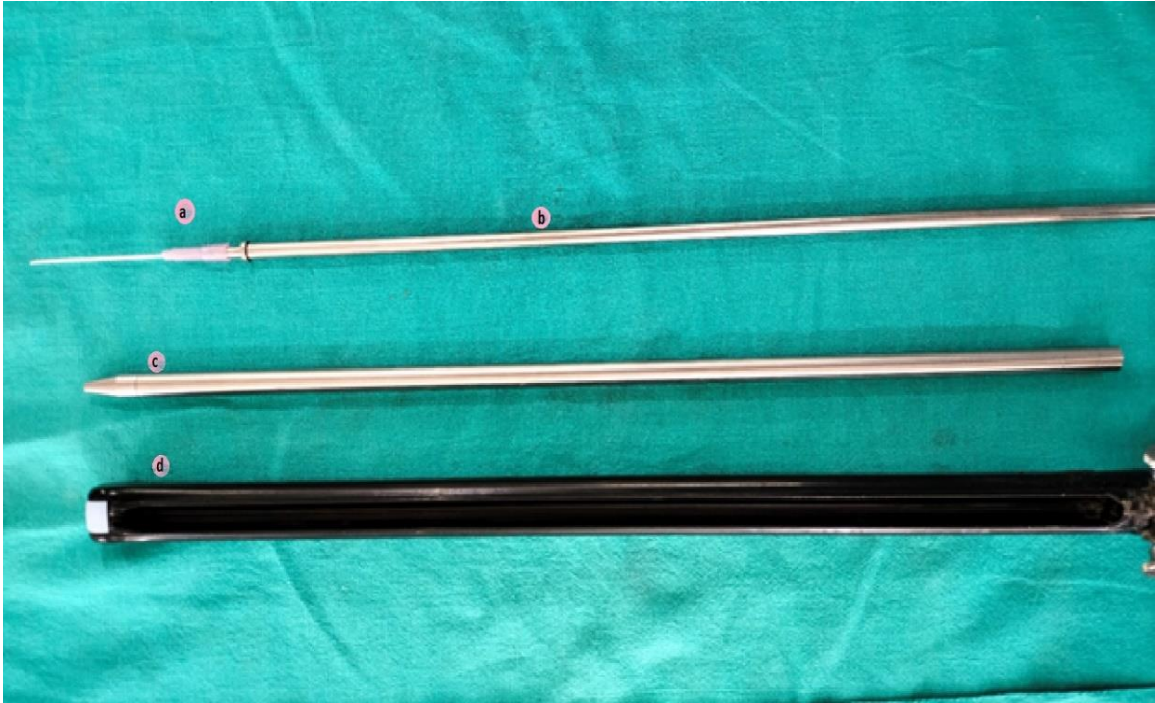


Plate 3: (a) Aspiration needle, (b) Aspiration line carrier, (c) needle guide and (d) transvaginal probe

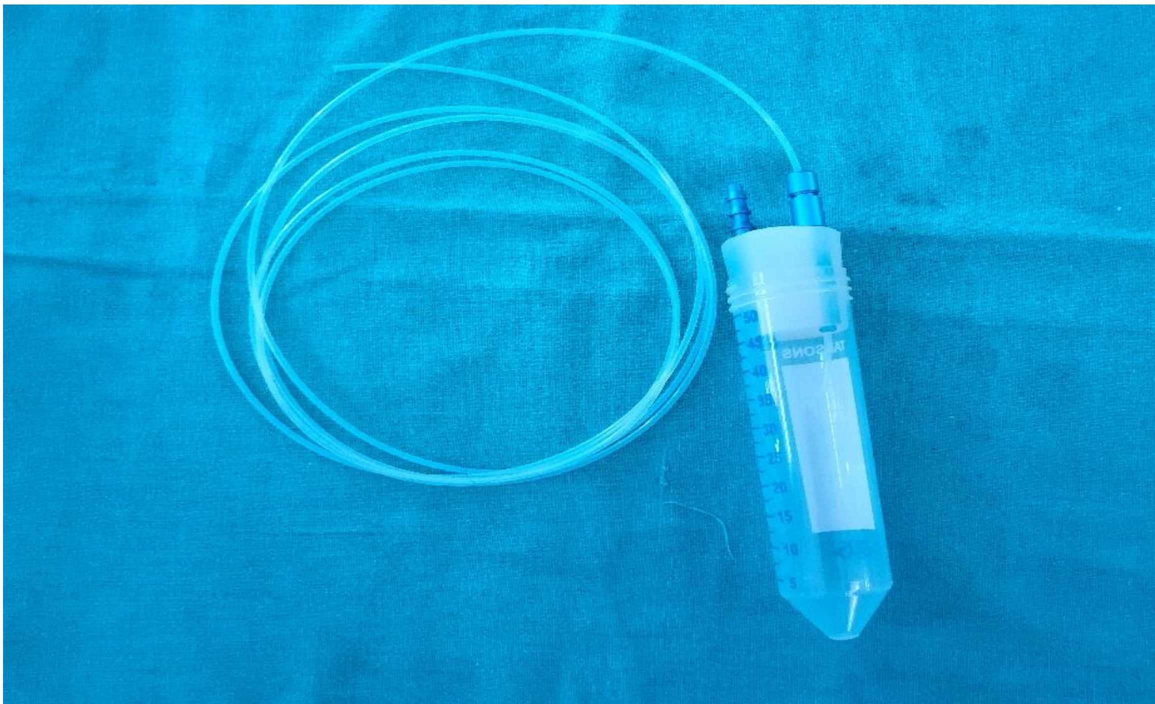


Plate 4: Aspiration line and 50ml centrifuge tube

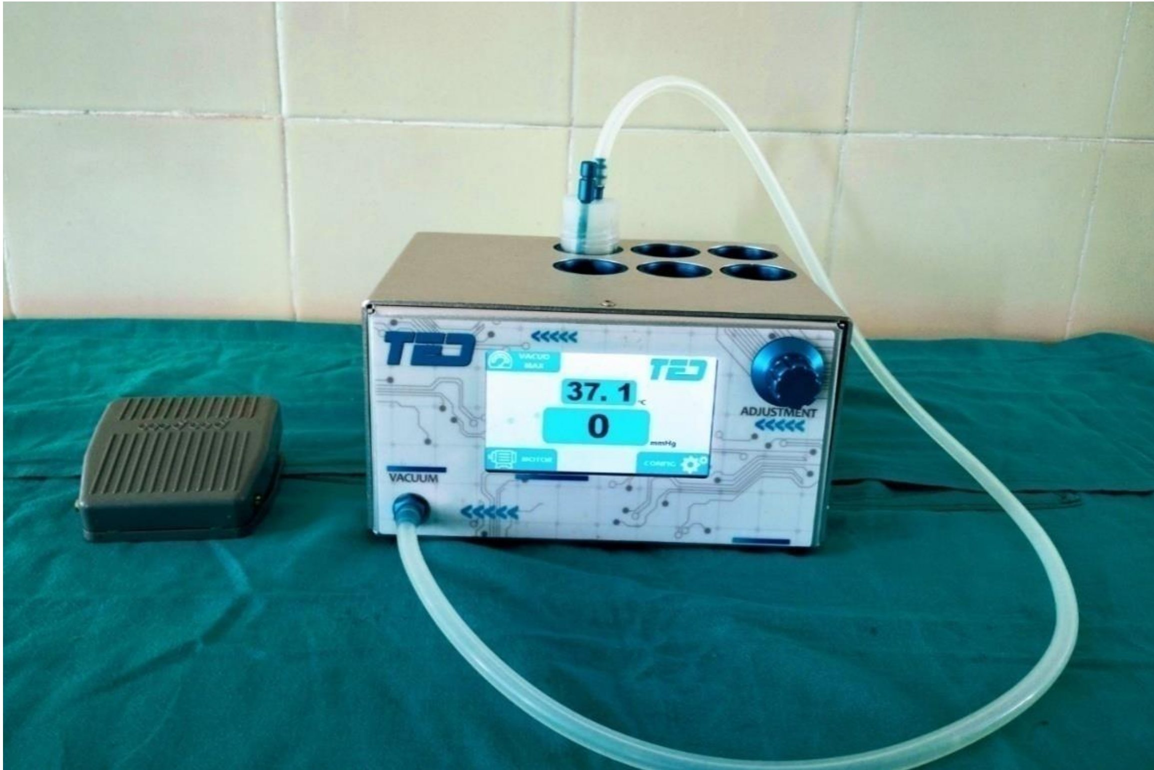


Plate 5: Vacuum pump with foot operated pedal switch

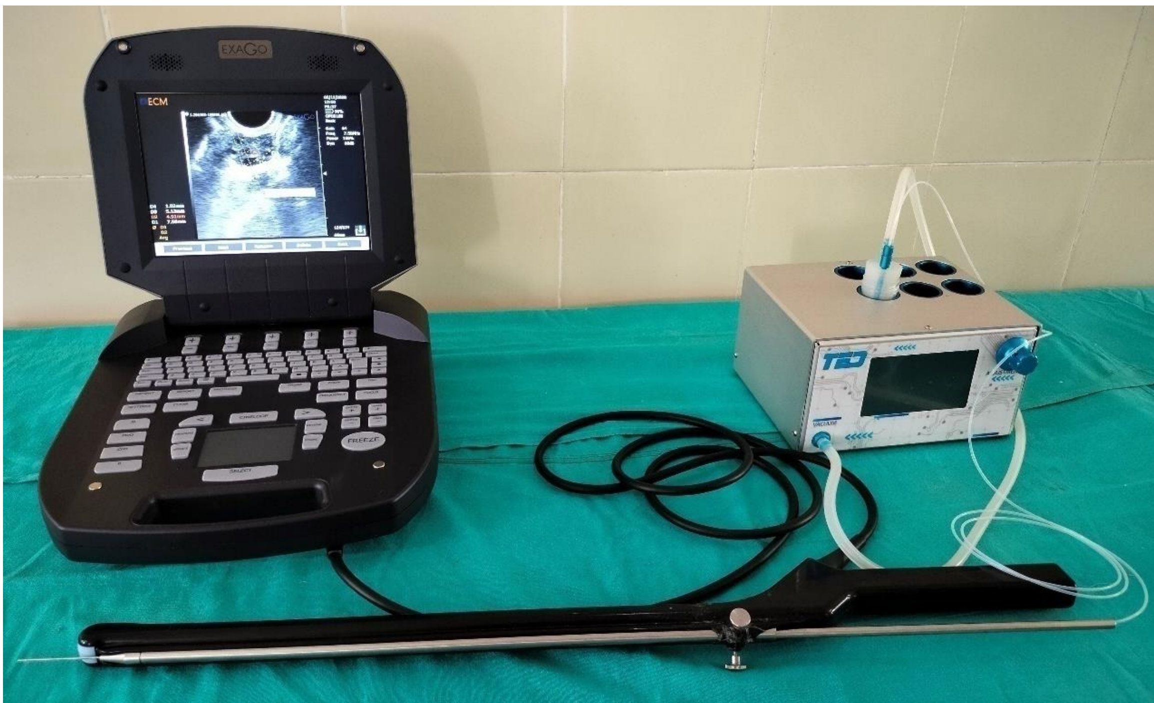


Plate 6: OPU collection unit (USG scanner, Transvaginal probe and Suction pump)

3.3.2 Donor preparation

To perform OPU, the OPU collection unit (Plate 6) was set and the animal was restrained in an adjustable squeeze chute. By back raking, the rectum was emptied taking care not to allow aspiration of air. Caudal epidural anesthesia was induced by administering 3-5ml of 2% Lignocaine Hydrochloride to prevent defecation, abdominal straining and to facilitate easy handling of the ovaries through rectum. The vulva and perineal area was washed with plain water, cleaned and dried with a sterilized napkin. The tail was held away and tied to one side with a cotton rope. The vulval lips were mopped with tissue paper soaked in 70% alcohol before insertion of vaginal probe.

3.3.3 Ovum pick-up technique (OPU)

The ovaries were manipulated per rectum and either the right or left ovary was positioned between the fingers. After thorough cleaning, the lubricated (Ultrasonic coupling gel) transvaginal probe with glove was advanced into the anterior vagina as far as possible, with the transducer surface in a position either to the left or to the right side of the external os of the cervix. Ultrasound scanning was done using 7.5 MHz frequency, 64-72 gain and 56% brightness.

The ovary was manipulated gently and positioned against the probe head in order to obtain a clear image of the follicles on the ultra-sonographic monitor. The number of follicles per ovary was recorded and the diameter of the follicles (Plate 7) was measured by freezing the image on the monitor and by using an inbuilt calliper and then the diameters of follicle was calculated by averaging the lengths of long and short axes (Nagai *et al*., 2015). Based on the diameter, the follicles were classified as small (<4mm), medium

(4 - <8mm) and large (≥8 mm) (Ginther *et al*., 1989). The presence of CL on the ovary and its diameter was also recorded.

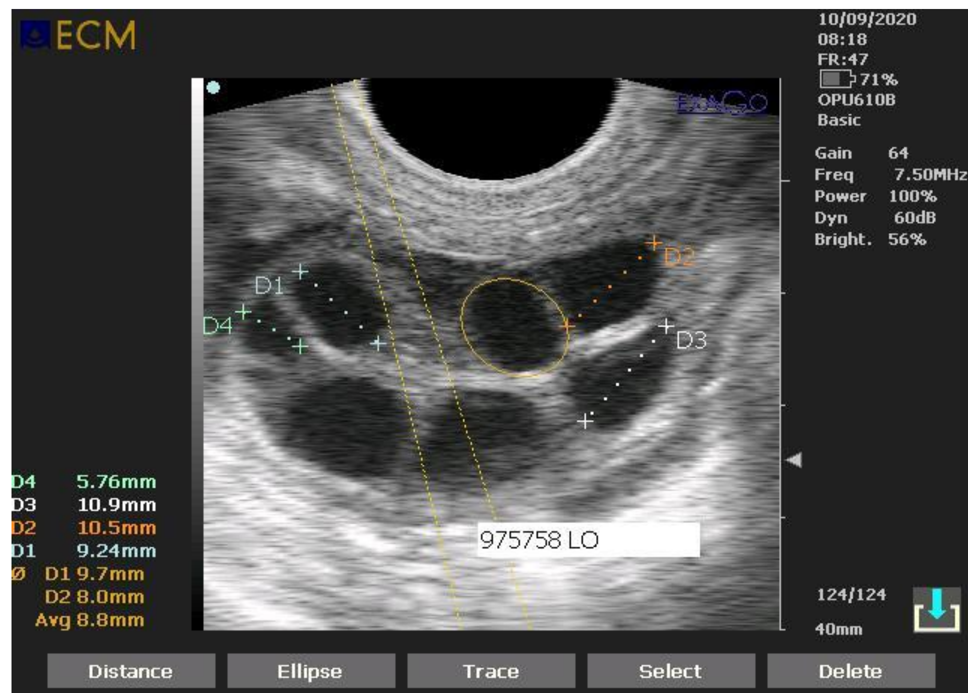


Plate 7: Ovary showing follicles of various sizes

When the ovary and targeted follicle were stabilized, the needle fitted with aspiration line was inserted through the plastic probe carrier and advanced to reach the fornix vagina and into the follicle antrum. Follicular fluid of each follicle was aspirated using continuous negative pressure of 70-75 mmHg, applied with a foot operated suction pump. During aspiration the needle was gently rotated to curette the follicle and to dislodge the oocyte still attached to its wall or trapped in its folds. Prior to aspiration of next follicle the needle was withdrawn from the ovary, but kept in position exterior to the fornix vagina with the tip still visible on the monitor. Before and after OPU, the needle and aspiration line were thoroughly rinsed with pre heated (37^{0}C) OPU recovery medium (Catalog

no.19982/1281, BoviPlus, Minitube, USA) to prevent blood from clotting or oocytes from sticking to the tubing.

The oocyte recovery rate was calculated as the number of oocytes recovered from the number of follicles aspirated for each cow expressed as a percentage (Goodhand *et al*., 2000).

During each aspiration, all visible follicles of >4mm diameter were aspirated and the contents were collected in a 50ml tube. A successful aspiration was confirmed by the disappearance of individual fluid - filled (non-echogenic and dark) follicle image on-screen display and the process was repeated until all the suitable follicles were aspirated. After aspiration of all follicles on one ovary, the same procedure was repeated in the opposite ovary. A separate needle was used for each donor.

3.4 Oocyte recovery

After completion of the aspiration of the each ovary, the 50mL conical centrifuge tube containing the follicular aspirate was transferred to the laboratory. The follicular aspirate was transferred to a 100 µm oocyte mini filter (25458, WTA, Brazil) and repeatedly washed with OPU recovery medium (Catalog no.19982/1281, BoviPlus, Minitube, USA) in order to make the filtered aspirate free from blood tinge and cloudy follicular fluid. The washed and filtered follicular aspirate was then transferred to a square grid petridish 90 x 15mm (Catalog no.150360, Thermo scientific, Massachusetts, USA) and examined under zoom stereomicroscope (SMZ - 1270, Nikon, Japan) at 1x magnification to identify the cumulus oocyte complexes (COCs). The COCs were transferred to a 35mm petridish containing WASH medium (BO 01, oocyte and embryo

wash media, Vitrogen, Brazil) and examined under stereozoom microscope at 8x magnification for evaluation and grading.

3.5 Evaluation of cumulus oocyte complexes

Generally the morphological evaluation and classification of cumulus oocyte complexes was based on the oocyte integrity, homogeneity of cytoplasm and the quantity of the cumulus cell layer surrounding the oocyte. During evaluation, the cumulus oocyte complexes were classified into four quality grades -Very good (Plate 8), Good (Plate 9), Fair (Plate 10A) and Poor (Plate 10B) according to Petyim *et al.* (2003).

3.6 *In-vitro* maturation of oocytes (IVM)

The oocytes were washed 4-6 times with Wash medium (BO 01, oocyte and embryo wash media, Vitrogen, Brazil) and then washed twice with IVM (BO 02, Vitrogen, Brazil). Cumulus-oocyte complexes were transferred into a CO2 equilibrated IVM well and kept for 24 hrs in a humidified CO_2 incubator (5% CO_2 in air) at 38.5°C.

After 24hrs of IVM of oocytes, assessment of maturation was done by the degree of expansion of cumulus cell mass (Plate 11) and extrusion of first polar body (Plate 12) into the perivitelline space.

3.7 *In vitro* fertilization of matured oocytes (IVF)

Previous day to *in vitro* fertilization, IVF media was prepared by adding 33µl H aliquot (BO 09, Vitrogen, Brazil), 132µl PHE aliquot (BO 10, Vitrogen, Brazil) and 2835µl IVF (BO 03, Vitrogen, Brazil) to make a total volume of 3 ml of IVF media. IVF dish was prepared with 35µl drops of IVF media. It was overlaid with mineral oil (BO 11,

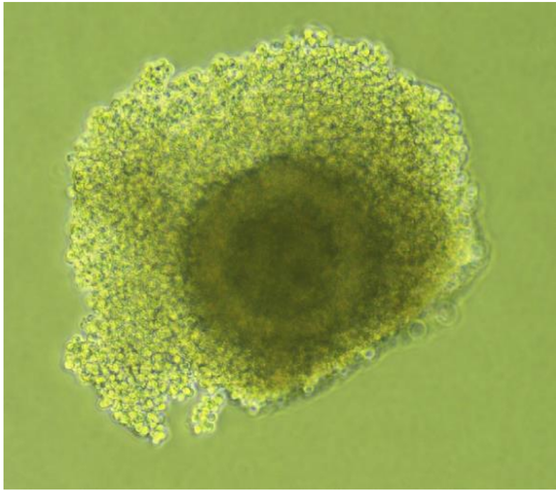


Plate 8: Grade 1 Oocyte

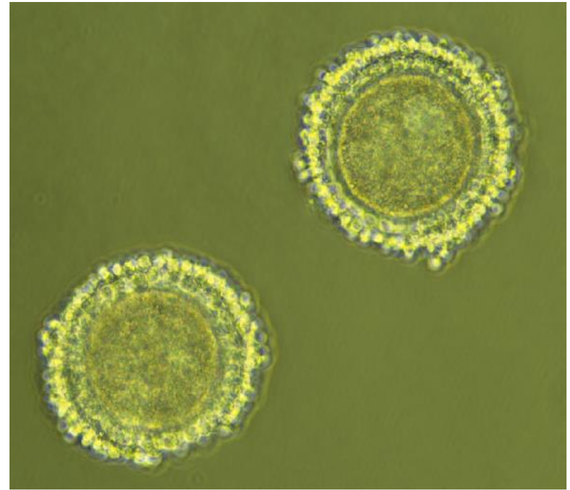


Plate 9: Grade 2 oocytes

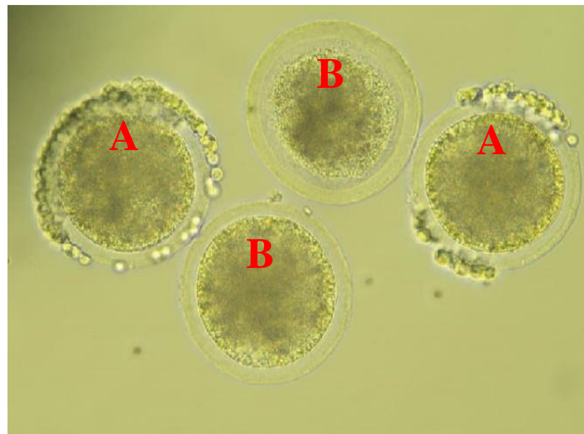


Plate 10: Grade 3 (A) and Grade 4 (B) Oocytes

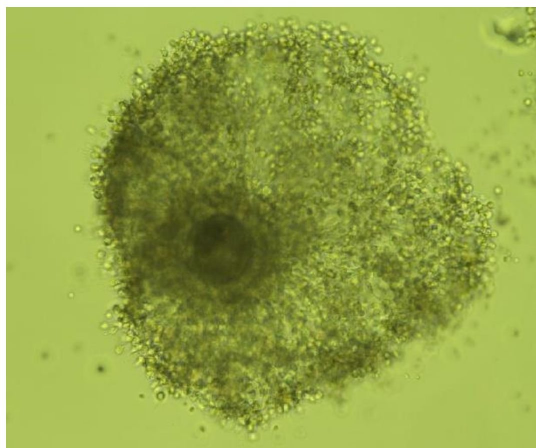


Plate 11: Cumulus cell expansion

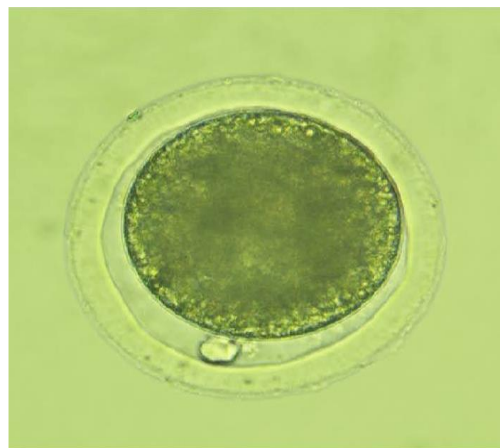


Plate 12: Extrusion of first polarbody

Vitrogen, Brazil) till all the drops were dipped/ submerged into the oil. Then, again 35µl of IVF media was added to the prepared IVF dish and kept in benchtop incubator (Planer incubator BT37, Ref. 026412, IMV Technologies, USA) at 38.8°C, in a humidified atmosphere of 6 % CO_2, 5 % O_2, and 89 % N_2 overnight.

On the day of IVF, after 24 hrs of IVM, matured oocytes were washed 2-3times with prepared IVF media and were then transferred into the drops of pre-equilibrated IVF media. The IVF dish containing matured oocytes was placed in the benchtop incubator till the semen was prepared.

Semen straw was thawed in thawing machine at 37°C for 30 seconds and was emptied into a 15ml falcon tube containing pre-warmed percoll gradient [400µl of conventional percoll (BO 07, Vitrogen, Brazil) + 400µl of diluted percoll (BO 06, Vitrogen, Brazil)] and centrifuged at 600G for 6min. at 37°C. The supernatant was removed leaving the pellet. To this pellet 400µl of prepared IVF media was added and centrifuged at 150G for 3min. at 37°C. The supernatant was again removed leaving the pellet and semen of approx. 10-20µl from the semen pellet was inseminated into IVF drops containing matured oocytes (Plate 13). The IVF dish was placed in benchtop incubator (Planer incubator BT37, Ref. 026412, IMV Technologies, USA) at 38.8°C, in a humidified atmosphere of 6 % CO_2, 5 % O_2, and 89 % for 16-18 hrs.

3.8 *In vitro* culture (IVC)

After 16-18hrs of co-incubation of gametes, the presumptive zygotes were transferred into the freshly prepared drops of preheated Wash media Putative zygotes were mechanically denuded of cumulus cells by repeated pipetting with denudation pipette in

Wash media. The denuded zygotes were washed 2-3 times with Wash media and later 2-3 times with preheated IVC media (BO 04, Vitrogen, Brazil). Then these zygotes were immediately transferred into pre-equilibrated IVC dish overlaid with mineral oil (Plate 14). *In vitro* culture dish was then kept in benchtop incubator (Planer incubator BT37, Ref. 026412, IMV Technologies, USA) at 38.8°C, in a humidified atmosphere of 6 %CO_2, 5 %O_2, and 89 % N_2 for 6 days. On day 6 and 7, development of blastocyst was observed.

3.9 Experimental design

The embryos of Day 6 and 7 (Plate 15 and 16) were randomly distributed among experimental groups as shown in Fig. 1. Fresh /Non-cryopreserved IVP embryos were used as the control group (Group 1).

3.9.1 Control group (Group 1)

The media IVC-ET (Embryo Transfer Medium, BO 05, Vitrogen, Brazil) was kept overnight in incubator to equilibrate and stabilize the pH. The blastocyst of Day 6 and 7 were washed 2-3 times with pre-equilibrated IVC-ET media to remove the overlaid oil. Then the embryos were loaded in a 0.25 ml sterile embryo straw between 2 air bubbles (Plate 17 and 18) and the straw was sealed with plug. One embryo per straw was loaded with identification mark on straw and the presence of embryo was observed (Plate 19 and 20). The embryo straws were kept in TED Embryo conveyor (TC- 39/240 OLED, ref-503008, Brazil) to maintain 37.5°C temperature (Plate 21 and 22) and were transferred to the recipient animal having functional corpus luteum and are in the same cycle as of donor (6-7 days after estrus). Forty days after the transfer, pregnancy was confirmed by rectal palpation and with transrectal ultrasonography.

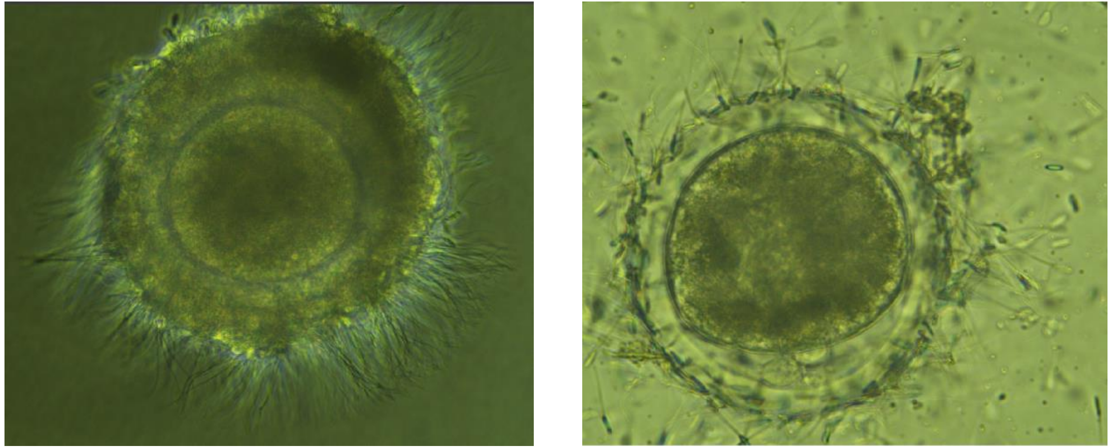


Plate 13: Co-incubation of oocyte and sperm

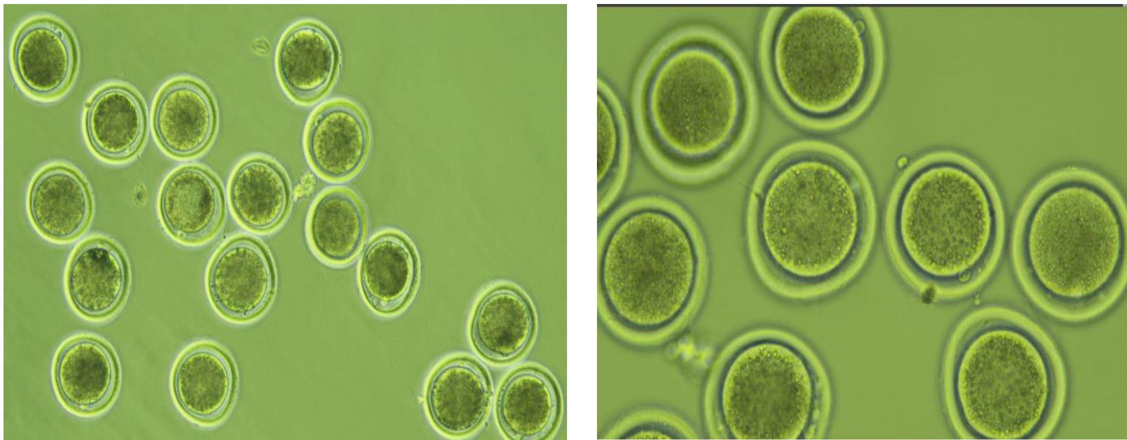


Plate 14: Presumptive zygotes in IVC media after denudation

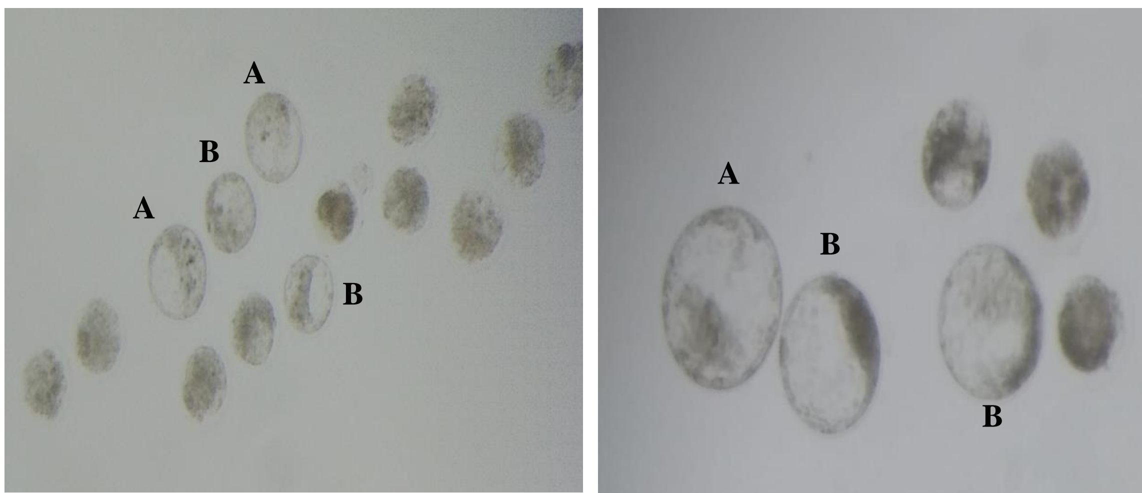


Plate 15: Embryos observed under stereozoom microscope (A) Expanded blastocyst and (B) Blastocyst

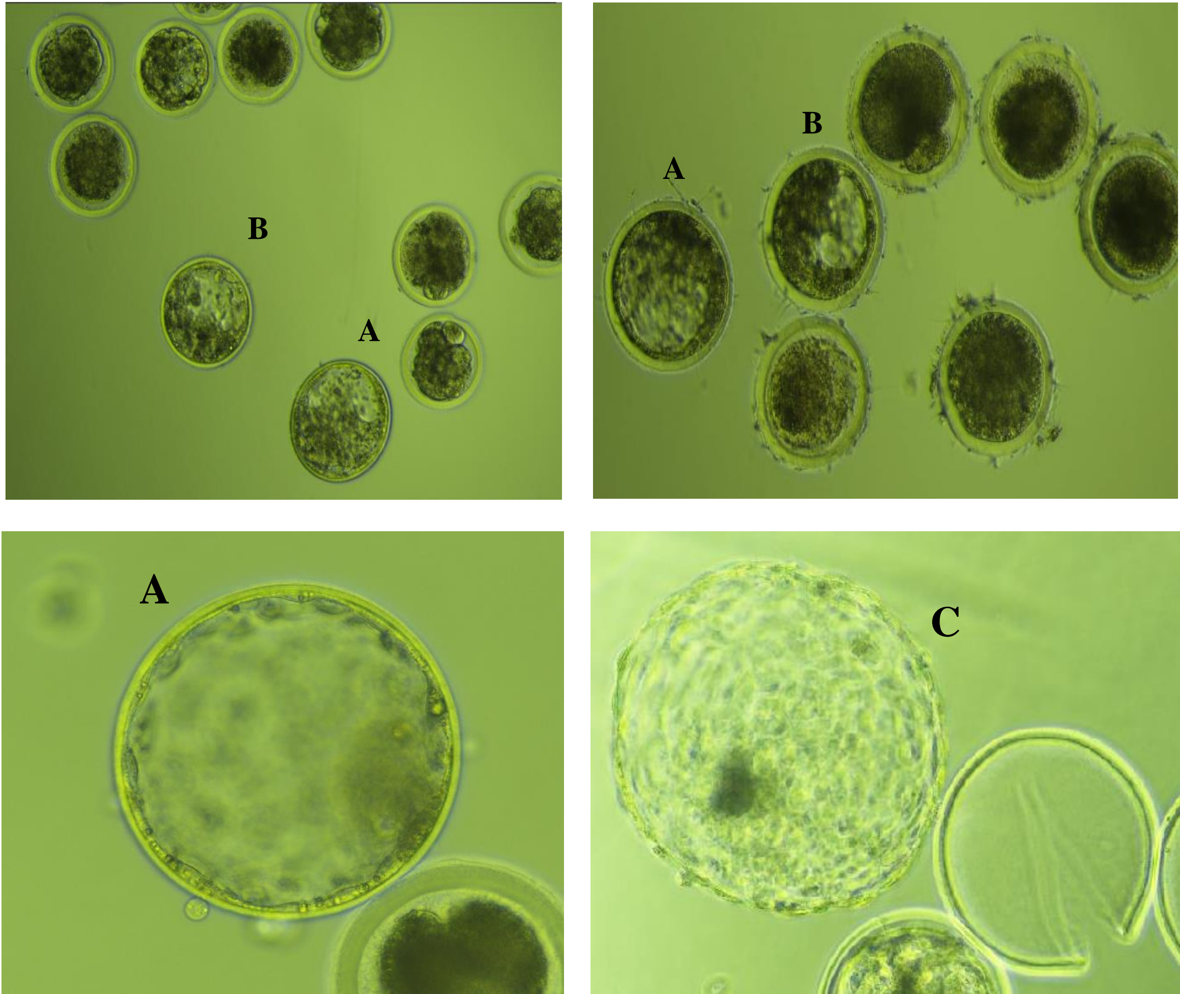


Plate 16: Embryos observed under phase contrast microscope. (A) Blastocyst, (B) Expanded blastocyst and (C) hatched blastocyst

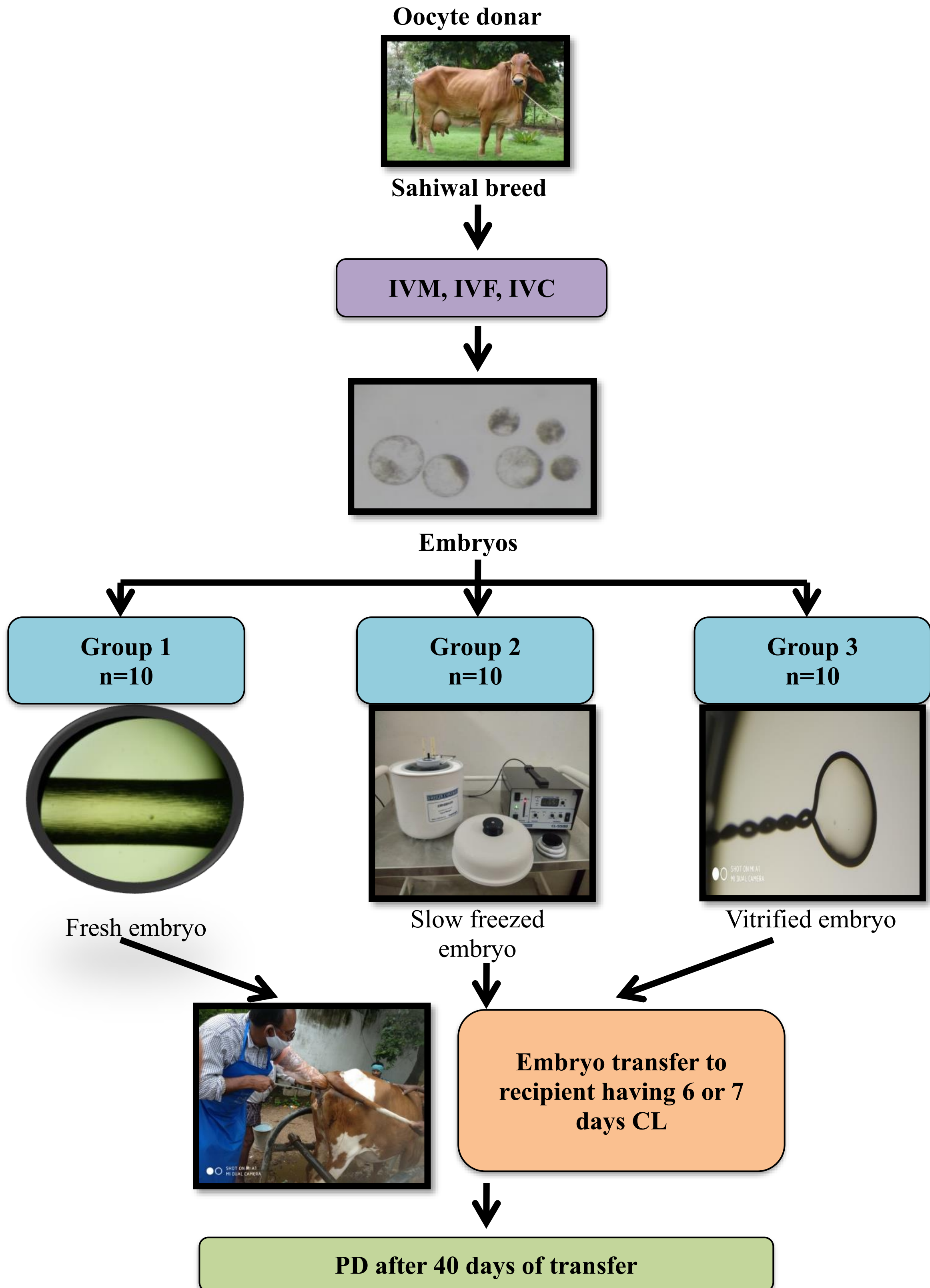


Fig. 1: Design of the study

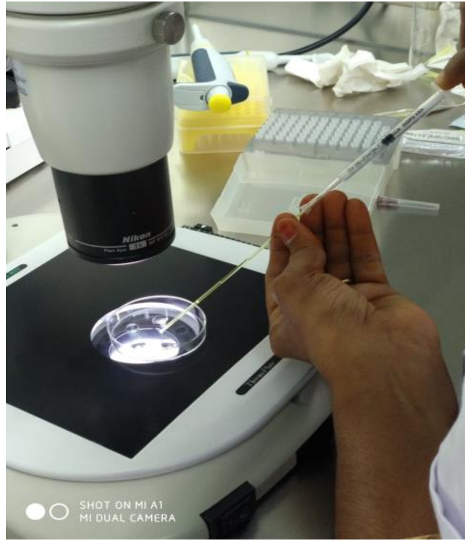


Plate 17: Embryo loading in 0.25ml straw

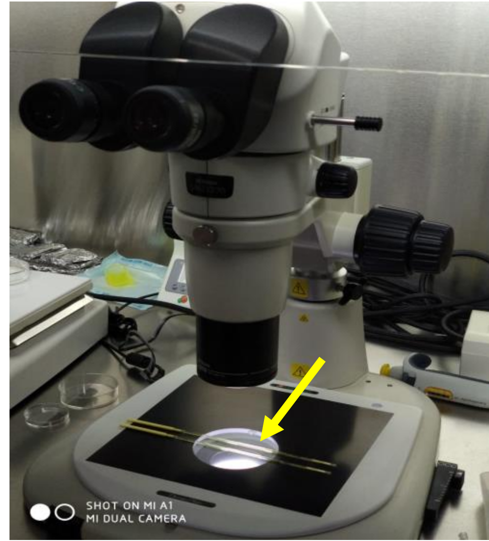


Plate 18: Loaded embryo straws

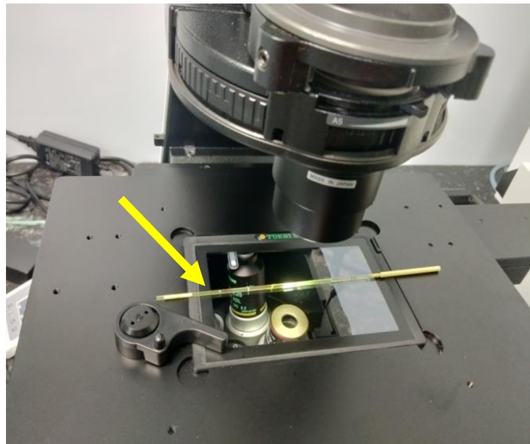


Plate 19: Observing embryo in the straw under phase contrast microscope

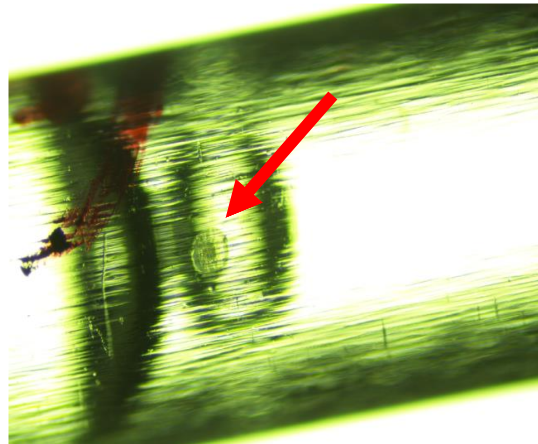


Plate 20: Embryo in the straw

Plate 21: TED Embryo conveyor

Plate 22: Keeping straw in embryo conveyor

3.9.2 Embryo cryopreservation

3.9.2.1 Slow-freezing at 0.5 ºC/min and direct transfer (Group 2)

The programmable freezer (PS-385 Freeze control®, model CL5500, CryoLogic, Australia) was started in order to reach its initial temperature (-6°C). Till the freezer reached its initial temperature, good quality blastocysts of 6th and 7th day culture were transferred from its culture media to 75µl drop of wash media (BO 01, Oocyte and Embryo Wash Medium, Vitrogen, Brazil) and the embryo straws were labelled. Slow freezing was done in freezing media HDT and EG (BOV15, Freezing kit, Vitrogen, Brazil) and the freezing process was carried out in an environment with a temperature of 22- 25°C. The freezing media was removed from refrigerator just before carrying out the process. Then the embryos were placed into 75µl drop of HDT media and kept for 1 minute. After 1 minute the embryos were transferred to 2 drop of 50µl EG and kept for 5-10 minutes. Within 5-10 minutes the embryo straw filling and transferring it into programmed freezer was done.

The embryos were loaded in a 0.25 ml sterile embryo straw with one embryo per straw. The loading of straw was done as following - Wash media - air bubble – EG – air bubble - EG + Embryo – air bubble – EG – air bubble – HDT- sealing plug. Then straws were placed in programmable freezer (Plate 23 and 24) at −6°C and seeding (the upper part of the ethylene column + embryo) was done by touching the straw with cold forceps or cotton dipped in LN_2 and kept the straw for 10 min. at -6°C. The straws were then cooled from −6 to −35ºC at a rate of 0.5°C/min. After reaching the target final temperature of −35ºC, straws were finally transferred into LN_2.

3.9.2.1.1 Thawing of Slow freezed embryos and direct transfer

Thawing was accomplished by holding the frozen straw for 10 sec in air and 30 sec in a 35°C water bath. The embryos were then transferred to the recipient animal having functional corpus luteum and are in same cycle as of donor (6-7 days after estrus). Forty days after the transfer, pregnancy was confirmed by rectal palpation and with transrectal ultrasonography.

3.9.2.2 Vitrification (Group 3)

Vitrification was done with media V1 and V2 (BOV13, Vitrification Media, Vitrogen, Brazil) at room temperature. Sixth and seventh days, good quality embryos were washed 2-3 times with culture media to remove overlaid oil. Then each embryo was placed into 50µl drop of V1 and kept for 8 minutes. After that, embryos were transferred into 50 µl drop of V2 and kept for 40 seconds. Within 40 seconds of transfer to V2 medium, the cryoloop was dipped in V2 media to form a thin film of V2 on it (Plate 25) and on that embryo was placed/ loaded (Plate 27). Once loaded with embryos, the cryoloop (Plate 26A) was plunged in to cryovial (Plate 26B) filled with LN_2 and screwed it. Then the cryovial was dipped in LN_2.

3.9.2.2.1 Devitrification

Devitrification was done with media D1, D2 and D3 (BOV14, Devitrification Media, Vitrogen, Brazil). Media D1 was warmed at 36-38°C and D2, D3 were kept at room temperature. The cryovial was removed from LN_2 tank. The cryoloop was unscrewed from cryovial and the loop was dipped in D1. The embryo was kept in D1 for 1minute. Then the embryo was transferred to D2 for 3minutes. After that it was transferred to D3 and kept for

Plate 23: Programmable freezer

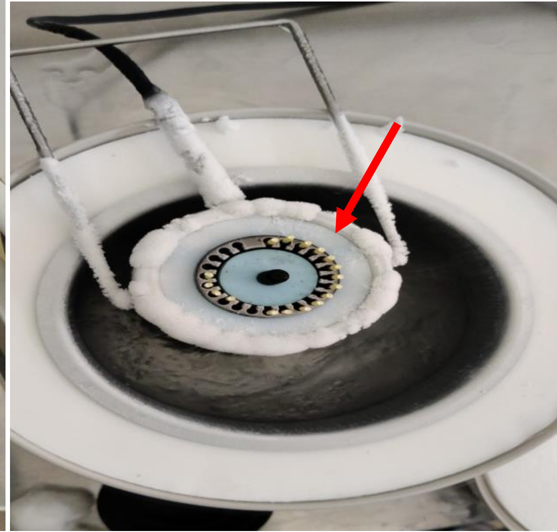


Plate 24: Straws in cryochamber

Plate 25: Loading embryo in cryoloop

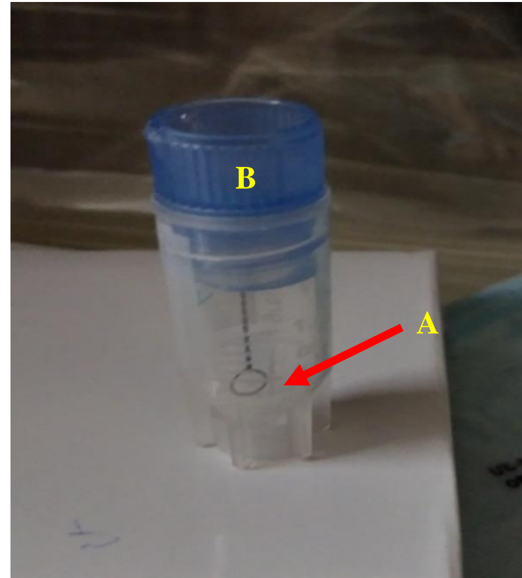


Plate 26: (A) Cryoloop in (B) cryovial

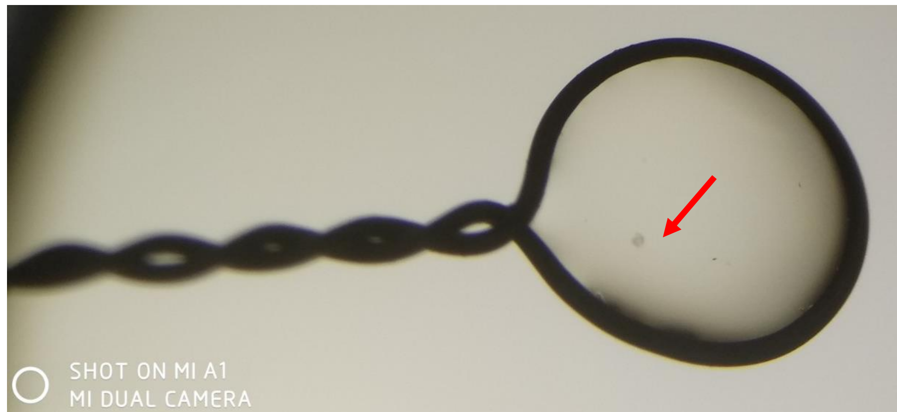


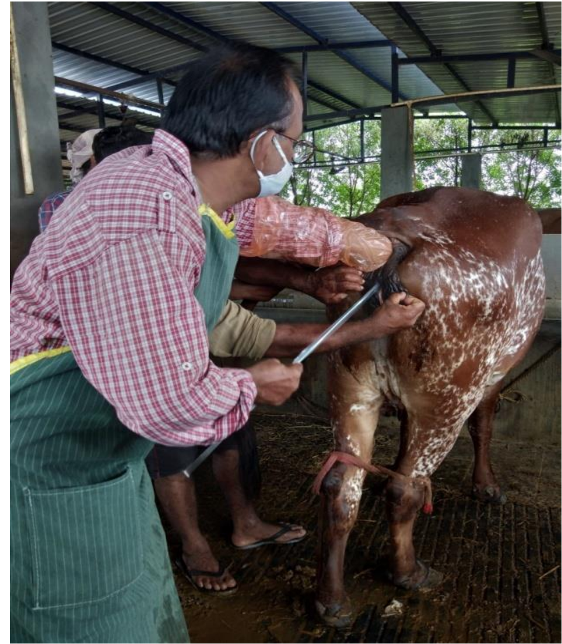
Plate 27: Embryo on thin film of vitrification media on cryoloop

5 minutes. After 5 minutes, the embryo was washed 2-3 times with IVC-ET media. Then the labelled sterile 0.25ml embryo straw was loaded with embryo between two air bubbles. The embryo straws were kept in TED Embryo conveyor (TC- 39/240 OLED, ref- 503008, Brazil) to maintain 37.5°C temperature and then transferred to the recipient animal having functional corpus luteum and is in the same cycle as of donor (6-7 days after estrus). Forty days after the transfer, pregnancy was confirmed by rectal palpation and with transrectal ultrasonography.

3.9.3 Embryo transfer procedure

All animals that have passed 6d of estrus, to them embryo transfers were performed non-surgically under epidural anaesthesia. Before embryo transfer (ET) healthy corpus luteum and its side was detected in recipient animal by using ultrasonography. Embryo Transfer was performed (Plate 28) into the uterine horn ipsilateral to the side where the CL was present. The embryo straw was loaded in the embryo transfer (ET) gun and the laboratory seal was cut. Embryo transfer gun was covered with ET sheath (IMV ET blue 5), which was then covered with ET sanitary sheath (IMV Chemises) and then embryo transfer was performed.

In each case, on day 40 after transfer, pregnancy status was confirmed (Plate 29) by rectal palpation and with transrectal ultrasonography (Easi-scan curve, BCF technology, Scotland, UK).



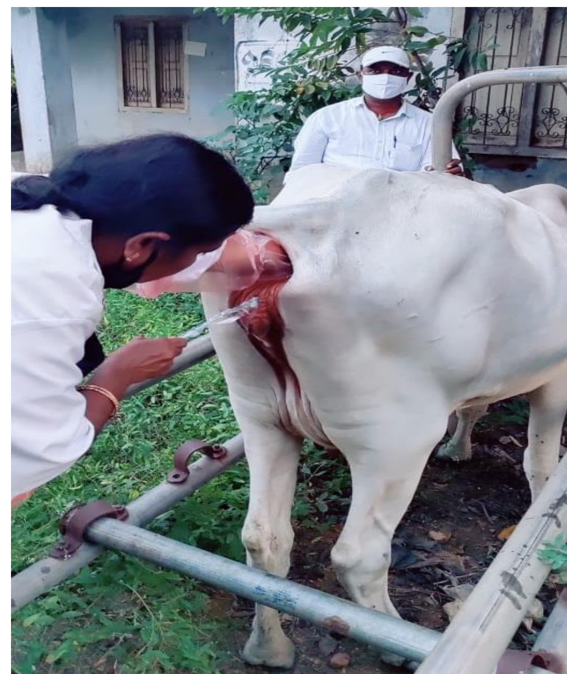


Plate 28: Embryo transfer to recipient

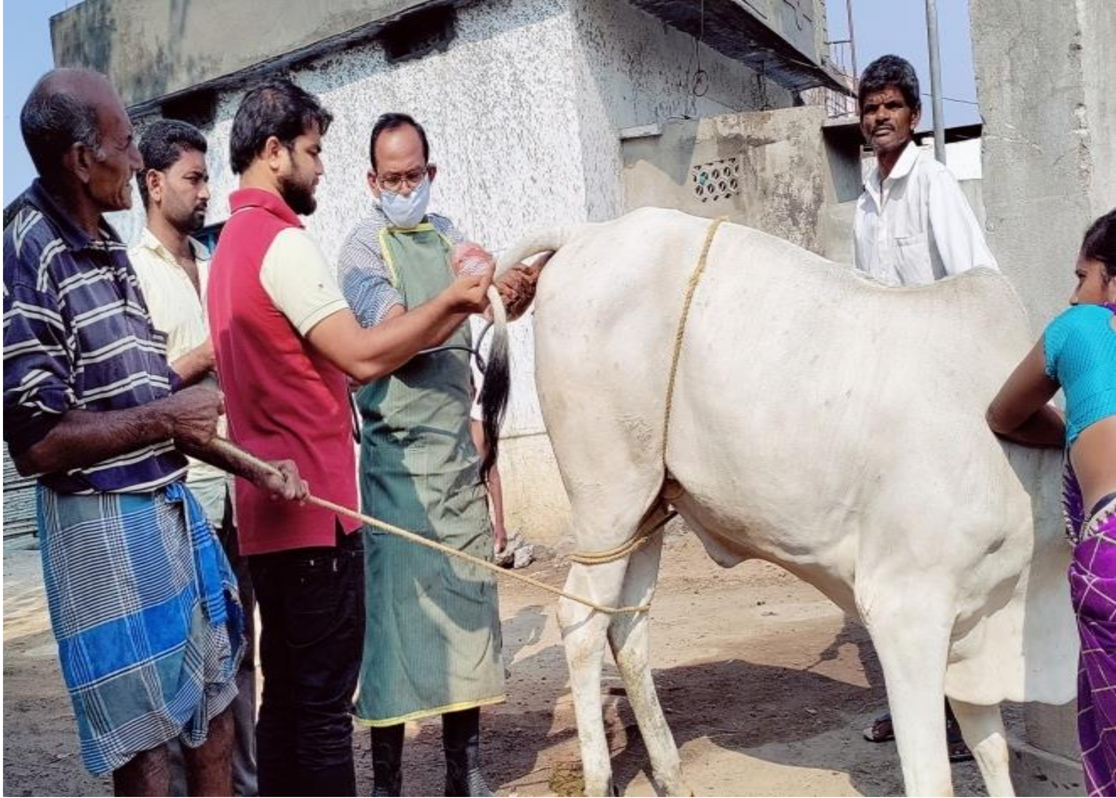


Plate 29: Pregnancy diagnosis using transrectal ultrasonography

Results

IV. RESULTS

The present experiment was conducted to compare the conception rate with fresh, slow freezed and vitrified bovine (Sahiwal) embryos produced through IVEP. Oocytes were collected through Ovum pick-up (OPU) method from Sahiwal cows maintained at Livestock farm complex, C.V.Sc, Korutla, Jagitial district, Telangana under the ET & IVF project (RGM). The collected oocytes were *in-vitro* matured, fertilized and cultured. 30 Good quality blastocysts produced after IVEP were randomly distributed among experimental groups- Group 1 (Fresh/ Non- cryopreserved embryos) n=10, Group 2 (Slow freezed embryos) n=10 and Group 3 (Vitrified embryos) n=10. These embryos were then transferred to the recipient having functional corpus luteum and in the same estrus cycle as of donor (6-7days of after estrus). Forty days after the transfer pregnancy was confirmed by rectal palpation and transrectal ultrasonography.

The results of this experiment that illustrate the conception rates with fresh, slow freezed and vitrified bovine (Sahiwal) embryos produced through IVEP are presented in this chapter.

4.1 Conception rate

The pregnancy after 40 days of transfer was confirmed by per rectal examination and transrectal ultrasonography Plate 30. Table No. 1 shows that the conception rates with fresh, slow freezed and vitrified IVP embryos were 3/10 (30%), 1/10 (10%) and 1/10 (10%) respectively.

The results of the study showed that the conception rate with IVP fresh/ non cryopreserved embryos (30%) was higher than slow freezed embryos (10%) and vitrified embryos (10%) which are depicted in Table no. 1. It also shows that there was no difference in the conception rates among 2 cryopreservation groups i.e IVP slow freezed (10%) and vitrified bovine embryos (10%). The results are also depicted in Fig.2.

Table 1: Results of conception rate with fresh, slow freezed and vitrified embryos

Embryo type	No. of embryo transfers done (n)	No. of animals pregnant (%)
Fresh / non cryopreserved	10	3 (30%)
Slow freezed	10	1 (10%)
Vitrified	10	1 (10%)

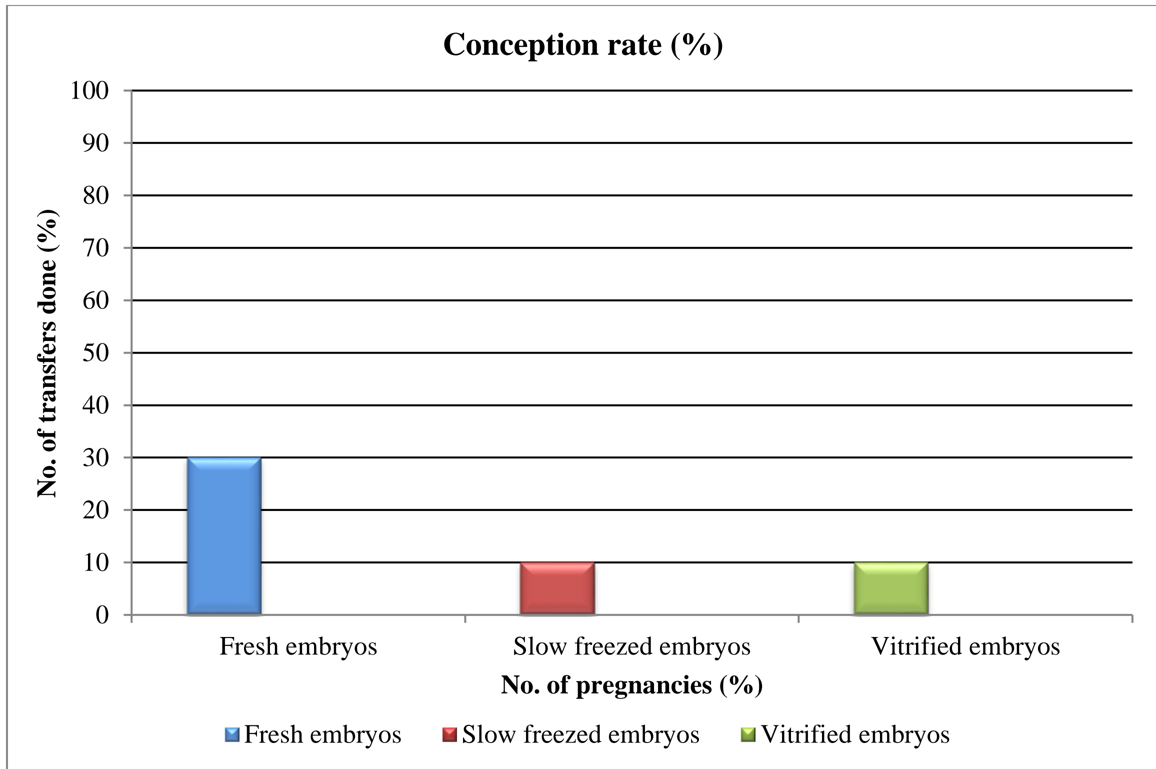


Fig. 2: Conception rate with Fresh, Slow freezed and vitrified IVP Sahiwal embryos

Plate 30: 40 days pregnancy

Discussion

V. DISCUSSION

The Sahiwal is one of the best dairy breeds in India and Pakistan due to its unique triats like tick-resistant, heat-tolerant, high milk production and noted for its high resistance to parasites, both internal and external. Out of total 192.49 million cattle population, total population of indigenous and nondescript cattle is estimated as 139.82 million, which shows a decline of 6 % in the total Indigenous (both descript and non-descript) cattle population over the previous census (Livestock census, 2019). This decline emphasizes the need for developing long term strategies for its conservation. Ovum Pickup – *Invitro* Embryo Production (OPU – IVEP) in combination with embryo cryopreservation and Embryo Transfer (ET) can be a viable alternative to conserve breeds, increase milk production and at the same time ensure faster multiplication of superior germplasm.

The application of Ovum Pick-up (OPU) technology, together with multistep embryo production *in vitro* (IVEP), represents a valid procedure for the recovery of oocytes from live donors and the attainment of a large number of embryos (Galli *et al.*, 2001). Despite the advantages offered by IVEP, the major challenge to its greater dissemination is to improve embryonic survival after cryopreservation. Embryo cryopreservation process is the most challenging aspect of embryo biotechnology, and despite advances in recent years, the results are still inconsistent (Sudano *et al.*, 2013). Cryopreservation methods aim to avoid the formation of intracellular ice crystals and to decrease the toxic effects generated by the cryoprotectant agent, minimizing the osmotic stress to the cells (Pryor *et al.*, 2009).

The total embryo production can sometimes be higher than the number of embryos to be transferred, so investment in research is necessary to develop an efficient protocol for the cryopreservation of the remaining embryos in a program (Sanches *et al.,* 2016). Therefore the present study was aimed to study and standardize the embryo cryopreservation methods in Sahiwal cow embryos and its effect on conception rate in recipient cows. In the present experiment, oocytes were collected from Sahiwal cows by ovum pickup method, which were then *in vitro* matured, fertilized and cultured. The IVP embryos were then randomly divided into the experimental groups- Group 1 (Fresh/ Non-cryopreserved) n=10, Group 2 (Slow freezed) n=10 and Group 3 (Vitrified) n=10. These embryos were then transferred to recipient animals having functional corpus luteum and are in the same cycle as of donors (6-7 days after estrus). Forty days after the transfer, pregnancy was confirmed by rectal palpation and transrectal ultrasonography. The results of conception rate obtained in this experiment with different group of embryos are discussed in this chapter.

5.1 Conception rate

5.1.1 Fresh embryos

In present study, the conception rate with IVEP fresh bovine embryos was 30%, which was in agreement with the study of Ismirandy *et al.*(2020), who reported that the recipient pregnancy rate of Bali cattle using fresh embryos was 41% (5/12). Similarly Do *et al.* (2018) showed that the pregnancy rate in recipient animal with IVEP derived fresh embryo transfer was 41.3%, which was similar as that of our results. Stewart *et al.* (2011) also reported similar results of about 42.1% conception rate with fresh embryos. On

contrary to the present study, Sanches *et al.* (2016) showed that the conception rates were 51.35±1.87% (133/259) with fresh embryos, which is higher than our results. Similarly, Gómez *et al.* (2020) showed that 40 days pregnancy rates with bovine fresh embryos were 63% (19/30). Block *et al.* (2010) showed that the percentage of cows pregnant with IVP fresh embryos were 56.3% (day 32) and 52.6% (d 46). However, Ambrose *et al.* (1999) had reported that the pregnancy rate 45 to 52d with IVP fresh embryos was 14.3% ± 2.3%, which is lower than our results. Similarly, Al-Katanani *et al.* (2002) showed lower pregnancy rate on day 45 with TET-Fresh was 19.0 ± 5.0%.

5.1.2 Slow freezed embryos

In present study, the conception rate with IVP slow freezed bovine embryos was 10%. In agreement with present study Ismirandy *et al.* (2020) reported similar results that the pregnancy rate of recipient Bali cattle with frozen embryos was 12.5% (1/8). On contrary to our results, Sanches *et al.* (2016) showed that the conception rate was 40.19±4.65% (125/311) for frozen embryos, which is higher than our results. Similarly, Gómez *et al.* (2020) showed that 40 days pregnancy rates with bovine frozen thawed embryos were 55% (22/40). Ambrose *et al.* (1999) had reported that the pregnancy rate 45 to 52d with IVP frozen embryos was 4.8% ± 2.3%, which is lower than the present study.

5.1.2 Vitrified embryos

In present study, the conception rate with IVP vitrified bovine embryos was 10%. On contrary to our results, Block *et al.* (2010) showed that the percentage of cows pregnant with IVP vitrified embryos were 33.1 % (day 32) and 29.6 % (d 46). Similarly, Stewart *et al.* (2011) reported that the percentage of cow pregnancy at d 40 ± 7 of gestation with the

ET-Vitrified was 29.3%. Sanches *et al.* (2016) showed that the conception rate was 35.89±3.87% (84/234) for vitrified embryos, which is higher than our results. Gómez *et al.* (2020) also showed a higher 40 days pregnancy rate with bovine vitrified embryos was 62% (29/47). Al-Katanani *et al.* (2002) showed that the pregnancy rate on day 45 was TET-Vitrified (6.5 ± 4.1%).

The lower conception rate in the present study could be due to the fact that 85% of the embryos transferred experience implantation failure and only 10%-15% show implantation success as stated by Febretrisiana and Pamungkas (2017). As reviewed and stated by Ferraz *et al.* (2016) the pregnancy per embryo transfer was affected by many factors like embryo type, embryo stage, embryo quality, recipient estrus cycle day at ET, (Temperature Humidity Index) THI, etc. These factors could have contributed for the present study results.

As stated by Ferré *et al.* (2020) IVP embryos are more sensitive to cryopreservation and is having lower cryotolerance than their *in vivo* counterparts, this could have led to the decreased pregnancy rate from cryopreserved embryos than fresh embryos in our study. In present study some of the ET was done in repeat breeders to improve pregnancy as concluded by Marinho *et al*. (2012) that the fixed timed embryo transfer (FTET) could result in the achievement of good pregnancy rates all year round, even in repeat-breeder cows and milk producing cows in the warmer months of the year. This could have added to the lower pregnancy rates in our study.

In conclusion, the present study showed a lower pregnancy rates when compared to other researchers on bovine embryos produced through IVEP (non-cryopreserved or

cryopreserved), which could be due to the beginning stage of embryo transfer work at experiment place during study, further it needs some time and practice to standardize and improve the pregnancy rates. Moreover the small sample size could be the reason for getting poor results. In future there is a scope for improvement. But from initial results we can conclude that fresh embryos give better pregnancy rates than slow freezed and vitrified embryos.

Summary

VI. SUMMARY

The application of Ovum Pick-up (OPU) technology, together with multistep embryo production *in vitro* (IVEP), represents a valid procedure for the recovery of oocytes from live donors and the attainment of a large number of embryos. Despite the advantages offered by IVEP, the major challenge to its greater dissemination is to improve embryonic survival after cryopreservation. Embryo cryopreservation process is the most challenging aspect of embryo biotechnology, and despite advances in recent years, the results are still inconsistent (Sudano *et al*., 2013). Cryopreservation methods aim to avoid the formation of intracellular ice crystals and to decrease the toxic effects generated by the cryoprotectant agent, minimizing the osmotic stress to the cells (Pryor *et al*., 2009).

The total embryo production can sometimes be higher than the number of embryos to be transferred, so investment in research is necessary to develop an efficient protocol for the cryopreservation of the remaining embryos in a program (Sanches *et al.,* 2016). Therefore the present study was aimed to study and standardize the embryo cryopreservation methods of Sahiwal cow embryos and its effect on conception rate in recipient cows.

The present study was conducted under the ET & IVF Project (RGM), Dept. Of VGO, College of Veterinary Science, Korutla, Jagitial district, Telangana. Oocytes were collected through Ovum pick-up (OPU) method from Sahiwal cows maintained at Livestock farm complex, C.V.Sc, Korutla, Jagitial district, Telangana. The collected oocytes were *in-vitro* matured, fertilized and cultured. Good quality blastocysts produced after IVEP were randomly distributed among experimental groups (Fig.1)- Group 1 (Fresh/

Non- cryopreserved embryos) n=10, Group 2 (Slow freezed embryos) n=10 and Group 3 (Vitrified embryos) n=10. These embryos were then transferred to the recipient animal having functional corpus luteum and are in the same cycle as of donor (6-7 days after estrus). Forty days after the transfer, the pregnancy was confirmed by rectal palpation and with transrectal ultrasonography.

The results of this experiment illustrate that the conception rate with IVP (bovine) Sahiwal fresh, slow freezed and vitrified embryos were 30% (3/10), 10% (1/10) and 10% (1/10) respectively and showed that the conception rate with IVP fresh embryos (30%) was higher than slow freezed (10%) and vitrified embryos (10%). The present study results also showed that there was no difference in conception rate with slow freezed (10%) and vitrified embryos (10%).

It can be concluded from the present study that the pregnancy rates were lower when compared to other researchers on bovine embryos produced through IVEP (non-cryopreserved or cryopreserved), which could be due to the beginning stage of embryo transfer work at experiment place during the study, further it needs some time and practice to standardize and improve the pregnancy rates. Moreover the small sample size could be the reason for getting poor results. In future there is a scope for improvement. But from initial results we can conclude that fresh embryos give better pregnancy rates than slow freezed and vitrified embryos.

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Abstract

VII. ABSTRACT

STUDY ON CRYOPRESERVATION OF EMBRYOS BY USING SLOW FREEZING AND VITRIFICATION METHODS IN SAHIWAL COWS

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The present study was conducted to compare the conception rate with fresh, slow freezed and vitrified bovine (Sahiwal) IVP embryos. Oocytes were collected through Ovum pick-up (OPU) method from Sahiwal cows maintained at Livestock farm complex, C.V.Sc, Korutla, Jagitial district, Telangana. The collected oocytes were *in-vitro* matured, fertilized and cultured. Good quality blastocysts produced after IVEP were randomly distributed among experimental groups- Group 1 (Fresh/ Non- cryopreserved embryos) n=10, Group 2 (Slow freezed embryos) n=10 and Group 3 (Vitrified embryos) n=10. These embryos were then transferred to the recipient having functional corpus luteum and are in same cycle as of donor (6-7 days after estrus). Forty days after the transfer, the pregnancy was confirmed by rectal palpation and with transrectal ultrasonography. The conception rate with IVP Sahiwal (bovine) fresh, slow freezed and vitrified embryos were 30% (3/10), 10% (1/10) and 10% (1/10) respectively. The results showed that the conception rate with fresh embryos (30%) was higher than slow freezed (10%) and vitrified embryos (10%). It also showed that there was no difference in conception rate with slow freezed (10%) and vitrified embryos (10%). When compared to other researchers, the results of present study was lower, which could be due to the beginning stage of embryo transfer work at experiment place during the study, further it needs some time and practice to standardize and improve the pregnancy rates. From present study results, it can be concluded that fresh embryos give better pregnancy rates than slow freezed and vitrified embryos. In future there is a scope for improvement.