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Effect of Cryopreservation on Conception Rates of *in-vitro* Produced Sahiwal Embryos

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ABSTRACT

Embryo cryopreservation process is the most challenging aspect of embryo biotechnology, and despite advances in recent years, the results are still inconsistent. Therefore, the present study was conducted to compare the conception rate with fresh, slow freezed and vitrified bovine (Sahiwal) embryos produced by *In vitro* embryo production (IVEP). Oocytes were collected through Ovum pick-up (OPU) method from Sahiwal cows maintained at Livestock Farm Complex (LFC), College of Veterinary Science, Korutla, Telangana, India under ET & IVF Project, Rashtriya Gokul Mission (RGM). The collected oocytes were *in-vitro* matured, fertilized and cultured. Thirty good quality blastocysts produced by IVEP were randomly distributed among experimental groups- Group 1 (Fresh/ Non- cryopreserved embryo) n=10, Group 2 (Slow freezed embryos) n=10 and Group 3 (Vitrified embryos) n=10. These embryos were then transferred to the recipient cows having a functional corpus luteum and were in sixth or seventh day of estrus cycle. Forty days after the transfer, the pregnancy was confirmed by transrectal palpation and with transrectal ultrasonography. The conception rate was 30%, 10% and 10% with IVEP fresh, slow freezed and vitrified embryos, respectively. It was concluded from the present study that the conception rate was higher with fresh embryos than with slow freezed and vitrified bovine (Sahiwal) embryos.

HIGHLIGHTS

- Conception rates are higher with fresh embryos when compared to slow freezed and vitrified embryos.
- Selecting Grade 1 embryos as per IETS manualfor transfer, gives good results.
- *In vitro* embryos are more cryosensitive than their *in vivo* counterparts.

Keywords: Conception rate, Embryo cryopreservation, IVEP, OPU, Sahiwal

India's total cattle population is 192.49 million, out of which total population of indigenous and nondescript cattle is estimated as 139.82 million, which shows a decline of 6% over the previous census (Livestock Census, 2019). This decline emphasizes the need for developing long

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term strategies for their 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 Indigenous breeds, increase milk production and it can increase the number of calves per unit of time by four to five times when compared to conventional ET (Gordon, 2004).

Despite the benefits provided by IVEP, improving embryonic survival following cryopreservation remains the challenging aspect (Sudano *et al*., 2013). Two factors determine cryopreservation protocols: the type and concentration of cryoprotectant and the rate with which cooling is done (Vajta and Kuwayama, 2006). Primarily the two commercial procedures for *in vitro* embryo cryopreservation are vitrification and slow freezing (traditional) (Saragusty and Arav, 2011). Cryopreservation techniques prevent the growth of intracellular ice crystalsand reduce osmotic stress on the cells (Pryor *et al*., 2009).

In slow freezing, programmable freezer is used to maintain a constant curve of cooling throughout the process, with the use of modest cryoprotectant concentrations since high concentrations are harmful to embryos but there is high risk of damage to organelles and membranes of embryo due to ice crystals formation (Dode *et al*., 2013). The slow freezing approach is more effective for commercial application when combined with the thawing and direct transfer (DT) of embryos to cows.

Vitrification is a simple, quick, and inexpensive cryopreservation method (Sanches *et al*., 2016). This technique dehydrates the embryonic cells and makes them permeable to the cryoprotectant by using a high-osmolarity solution that causes the embryonic intracellular water to depart quickly.As a result, the embryo can endure being submerged directly in liquid nitrogen (-196°C) without ice crystals formation (Kim *et al.*, 2012). Conversely, high concentration of cryoprotectant promotes substantial cellular toxicity, even when exposed for a brief time or small quantity of this solution is used (Fahy and Wowk, 2015). As a result, many methods, such as open pulled straw (OPS) (Bhat *et al.*, 2015), cryoloop (Kim *et al.*, 2012), microdroplets (Nguyen *et al.*, 2018), and cryotopapproaches (Gonzalez-Plaza *et al.*, 2022), have been developed to allow embryos to quickly come into contact with liquid nitrogen while also reducing the volume of the cryoprotectant agent.

The total embryo production can sometimes be higher than the 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 current study's objectives were to study embryo cryopreservation methods and compare the conception rate with fresh, slow freezed and vitrified *in vitro* Sahiwal embryos.

MATERIALS AND METHODS

The present study was undertaken at LFC, College of Veterinary Science, Korutla, under the ET & IVF Project (RGM), Dept. of VGO, CVS, Korutla, Telangana, India. Sahiwal cows (*Bos indicus*) aged three to six years were selected as oocyte donors for OPU. The research work and all procedures were approved by IAEC with No. 12/2020/VCB/VGO dated 13/3/2020.

Ovum pick up and *in-vitro* maturation (IVM)

OPU was performed under caudal epidural anesthesia, the follicular aspirate was sent to the laboratory and transferred to 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 clot and cloudy follicular fluid. The washed and filtered follicular aspirate was then examined under stereozoom microscope (SMZ - 1270, Nikon, Japan) at 10x magnification to identify the cumulus oocyte complexes (COCs). The COCs were transferred to a 35 mm petridish containing wash medium (BO, O_1, oocyte and embryo wash media, Vitrogen, Brazil) and examined under stereozoom microscope at 50x magnification for evaluation and grading of COCs. Grade 1- several layer of cumulus cells, Grade 2- oocytes with more than three-layers of cumulus cells, Grade 3- oocyte with one to two-layers of cumulus cells, Grade 4- oocyte with no cumulus cells (De Roover *et al*., 2005). All COCs from Grade 1 to 4 were selected and washed four to six times with Wash medium and then washed twice with IVM medium (BO O_2, Vitrogen, Brazil). Cumulus-oocyte complexes were then transferred into a CO_2 equilibrated IVM well and kept for 24 hrs in a humidified CO_2 incubator (5% CO_2 in air) at 38.5°C.

In vitro fertilization (IVF)

Preceding day to *in vitro* fertilization, IVF media was prepared by adding 33 µl H aliquot (BO 09, Vitrogen, Brazil), 132 µl PHE aliquot (BO 10, Vitrogen, Brazil) and 2835 µl IVF (BO O_3, Vitrogen, Brazil) to make a total volume of 3 ml of IVF media. IVF dish was prepared with 35 µl drops of IVF media and was overlaid with mineral oil (BO 11, Vitrogen, Brazil). Then, again 35 µl 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.

After 24 hrs of IVM, matured oocytes were washed two to three times with prepared IVF media, then transferred into the drops of pre-equilibrated IVF media and the IVF dish 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 15 ml falcon tube containing pre-warmed percoll gradient [400 µl of conventional percoll (BO 07, Vitrogen, Brazil) + 400 µl of diluted percoll (BO 06, Vitrogen, Brazil)] and centrifuged at 600G for 6 min. at 37°C. The supernatant was removed leaving the pellet, to which 400 µl of prepared IVF media was added and centrifuged at 150 G for 3 min. at 37°C. The supernatant was again removed leaving the pellet and semen of approx. 10-20 µl from the semen pellet was inseminated into IVF drops containing matured oocytes. The IVF dish was placed in benchtop incubator at 38.8°C, 6% CO_2, 5% O_2, and 89% N_2 for 16-18 hrs.

In vitro culture (IVC)

After 16-18 hrs of co-incubation of gametes, the presumptive zygotes were transferred into the freshly prepared drops of preheated Wash media. Putative zygotes were mechanically denuded by repeated pipetting with denudation pipette and were washed two to three times with Wash media and later two to three times with preheated IVC media (BO O_4, Vitrogen, Brazil). Then these zygotes were immediately transferred into pre-equilibrated IVC dish overlaid with mineral oil. *In vitro* culture dish was then kept in benchtop incubator at 38.8°C, 6% CO_2, 5% O_2, and 89 % N_2 for six days. On day six and seven, development of blastocyst was observed. Successive rate of each stage of IVEP are shown in Table 1.

Experimental design

The embryos of day six and seven (Fig. 1) with Stage

Table 1: Successive rate of each stage of IVEP

Total no. of oocytes recovered	No. of oocytes kept for IVM	No. of oocytes matured (%)	No. of oocytes kept for IVF	No. of oocytes cleaved (%)	No. of zygotes kept for IVC	No. of embryos formed (%)
329	327	115 (35.16%)	115	98 (85.21%)	98	85 (86.73%) [Fresh 18, Slow Freezed = 27, Vitrified = 40]

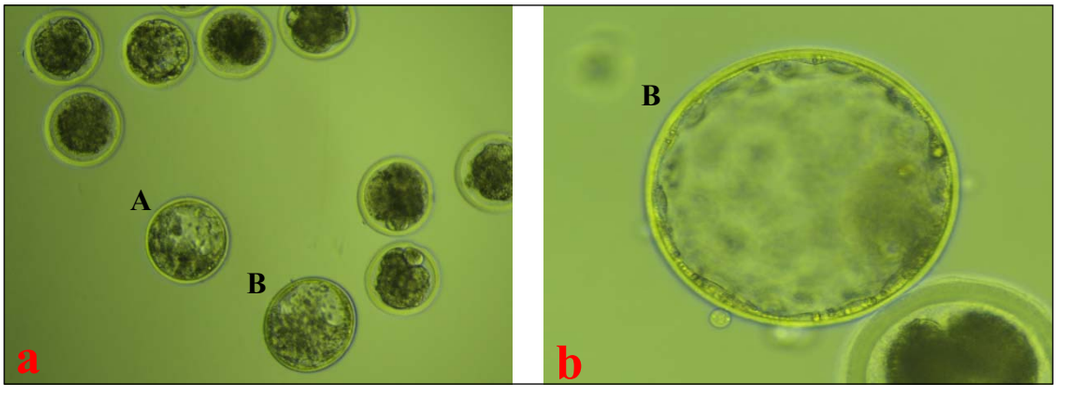

Fig. 1: a, b- Embryos observed under phase contrast microscope. **(A)** Blastocyst, **(B)** Expanded blastocyst

7 and Grade 1 as per IETS manual (Bó and Mapletoft, 2013) were selected and randomly distributed among experimental groups. Fresh/Non-cryopreserved IVP embryos were used as the control group (Group 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 six and seven were washed two to three times with pre-equilibrated IVC-ET media and the embryos were loaded in a 0.25 ml sterile embryo straw between two air bubbles 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 (Fig.2B). The embryo straws were kept in TED Embryo conveyor (TC-39/240 OLED, ref- 503008, Brazil) to maintain 37.5°C temperatureand were transferred to the recipient animal.

Embryo cryopreservation

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

The programmable freezer (Fig. 2A, PS-385 Freeze control®, model CL5500, CryoLogic, Australia) was switched on in order to reach its initial temperature (-6°C). Till the freezer reached its initial temperature, blastocysts of sixth and seventh day culture were transferred from its culture media to 75 µl drop of wash media and the embryo straws were labeled. 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 to 25°C. The freezing media was removed from refrigerator just before carrying out the process. Then the embryos were placed into 75 µl drop of HDT media and kept for 1 minute. After 1 minute the embryos were transferred to two drop of 50 µl EG and kept for 5 to 10 minutes. Within 5 to 10 minutes filling of the embryo straw and transfer into programmed freezer was completed.

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 freezerat −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 final temperature of −35ºC, straws were finally transferred into LN_2.

Thawing of Slow freezed embryos and direct transfer

Thawing was accomplished by holding the frozen straw for 10 seconds in air and 30 seconds in a 35°C water bath. The embryos were then transferred to the recipient animal.

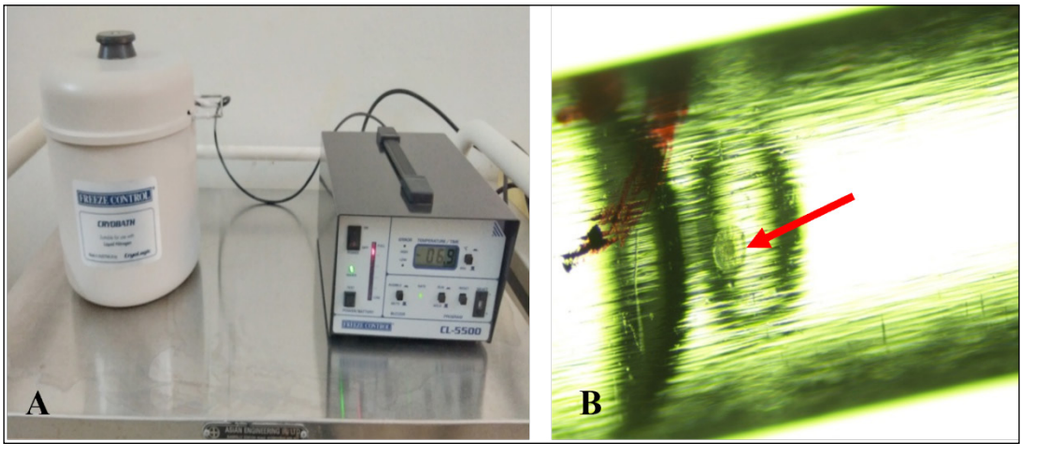

Fig. 2: (A) Programmable freezer and **(B)** Embryo in the straw (arrow)

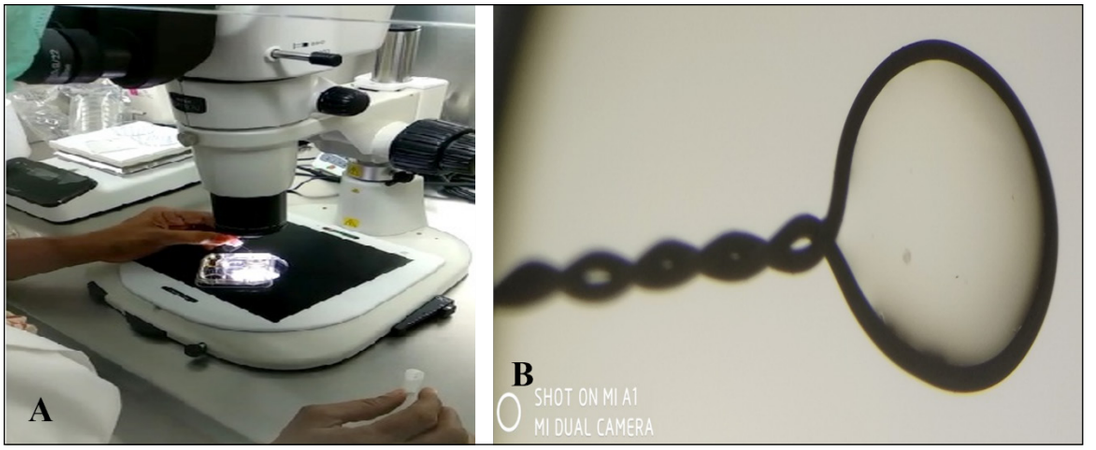

Fig. 3: (A) Loading embryo in cryoloop and **(B)** Embryo on thin film of vitrification media on cryoloop

Vitrification (Group 3)

Vitrification was done with media V1 and V2 (BOV13,Vitrification Media, Vitrogen, Brazil) at room temperature. Embryos of sixth and seventh day culture were washed two to three times with IVC media and were placed into 50 µl 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 (Fig. 3A) to form a thin film of V2 on it and on that embryo was placed/ loaded (Fig. 3B). Once loaded with embryos, the cryoloop was plunged in to cryovial filled with LN_2 and closed. Then the cryovial was dipped in LN_2.

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 1 minute and then transferred to D2 for 3 minutes, later to D3 and kept for 5 minutes. After 5 minutes, the embryo was washed two to three times with IVC-ET media. Then the labeled sterile 0.25 ml embryo straw was loaded with embryo between two air bubbles. The embryo straws were kept in TED Embryo conveyor to maintain 37.5°C temperature and then transferred to the recipient animal.

Embryo transfer procedure

All animals that were present on sixth or seventh-day of estrus cycle were selected and embryo transfers were performed non-surgically under epidural anaesthesia. Before embryo transfer (ET) a healthy corpus luteum and its side was detected by using ultrasonography. Embryo transfer was performed into the uterine horn ipsilateral to the side of CL. 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 was confirmed (Fig. 4) by transrectal palpation and transrectal ultrasonography (Easi-scan curve, BCF Technology, Scotland, UK).

RESULTS

The conception rates with fresh, slow-frozen, and vitrified IVP embryos and the number of animal parturated (Fig. 5) among conceived animals are shown in Table 2.

The study's findings demonstrated that the IVP fresh embryo conception rate (30%) was higher than the slow freezed embryo (10%) and the vitrified embryo (10%) conception rate. It also demonstrated that the conception rates of the two cryopreservation groups, namely slow freezed and vitrified bovine embryos, both stood at 10%.

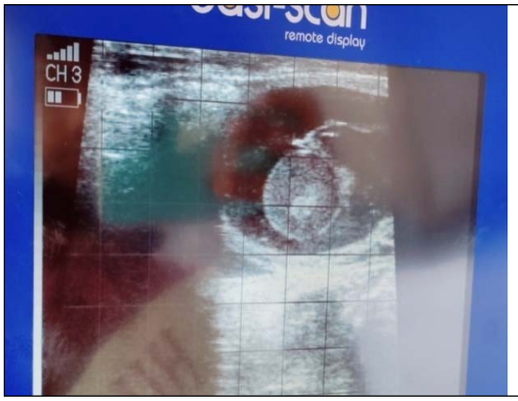

Fig. 4: 40 days pregnancy

Fig. 5: Parturated animals with calf

Table 2: Results of conception rate with fresh, slow freezed and vitrified embryos

Embryo type	No. of embryo transfers done (n)	No. of animals pregnant (%)	No. of animals parturated
Fresh	10	3 (30%)	1
Slow freezed	10	1 (10%)	1
Vitrified	10	1 (10%)	0

DISCUSSION

In the current study, the conception rates of fresh, slow-freezed and vitrified bovine (Sahiwal) embryos produced by IVEP were compared. According to Ferré *et al.* (2020), IVEP embryos are more cryosensitive and have poor cryotolerance than their *in vivo* counterparts, which may explain why a lower conception rate was observed in present study with cryopreserved embryos than fresh embryos. According to Nowshari and Brem (2001), physical injury, ice crystal formation during solidification, toxic effects of cryoprotectants and osmotic stress during cryoprotectant release all contribute to the reduction in the life span of embryos after cryopreservation.

The commercial production of in vitro bovine embryos has significantly increased over the past decade (Stroud andBó, 2011), however majority of them are still transferred fresh. One of the reasons is the abundance of potential recipients in nations like Brazil and Argentina (Stroud and Bó, 2011) and their higher survivability as compared to cryopreserved embryos. In present study, the conception rate with IVEP fresh embryos was in consistent with studies of Ismirandy *et al.* (2020), and Stewart *et al.* (2011). On contrary, Sanches *et al.* (2016), Gómez *et al.* (2020), and Block *et al*. (2010) demonstrated increased conception rates with fresh embryos. On the other hand, studies by Ambrose *et al.* (1999) and Al-Katanani *et al.* (2002) revealed reduced pregnancy rates.

The demand for IVEP has significantly increased on a global scale, largely due to the higher effectiveness of genetic improvement in dairy herds (Pontes *et al*., 2010), which gives emphasis to cryopreserved embryos. The most common cryopreservation technique combines slow programmable freezing with direct transfer of frozen-thawed embryos into the uterus, making it simpler and more practical at dairy farms (Sanches *et al*., 2016). In the current study, the findings were similar to Ismirandy *et al.* (2020). On contrary, Sanches *et al.* (2016) and Gómez *et al.* (2020) showed higher pregnancy rates with bovine frozen thawed embryos. However, Ambrose *et al.* (1999) had reported a lower pregnancy rate with IVEP frozen embryos.

In vitro embryo cryopreservation via vitrification is now the most popular method adopted globally, greatly enhancing the effectiveness of IVEP programmes and allowing embryo storage. According to studies, vitrification approach has a higher incidence of IVEP embryo survival than slow freezing (Villamil *et al*., 2012). The present findings are contrary to the results of Sanches *et al.* (2016) and Gómez *et al.* (2020), who revealed that bovine vitrified embryos had a greater 40-day pregnancy rate. However, Al-Katanani *et al.* (2002) showed a lower pregnancy rate on day 45 with TET-Vitrified embryos. According to Zárate-Guevara *et al.* (2018), a successful pre-treatment approach of Forced Blastocoele Collapse (FBC) or Blastocentesis can be carried out before slow freezing or vitrification of bovine blastocyst, to improve conception rate, as it guards against damage brought on by ice crystal formation during standard cryopreservation.

The overall lowered conception rate in present study when compared to other reports could be due to the fact that 85% of the transferred embryos experience implantation failure, and just 10%–15% exhibit implantation success (Febretrisiana and Pamungkas, 2017). As reviewed and stated by Ferraz *et al.* (2016) the pregnancy following an embryo transfer was influenced by factors like embryo type, embryo stage, embryo quality, recipient's stage of estrus cycle on the day of ET, (Temperature Humidity Index) THI, etc. During study, transfer of embryos was done in repeat breeders to improve pregnancy as suggested by Marinho *et al.* (2012) that fixed timed embryo transfer (FIET) improves pregnancy rates in repeat-breeder cows. This could have further added to the lowered pregnancy rates in the present study.

Other reasons for lower pregnancy rates could be the beginning stage of embryo transfer work at experiment place, during study. Further, experience and practice were also needed to standardize and improve the pregnancy rates. Moreover, the small sample size could be one of the reasons for getting poor results. In future there is a wide scope for improvement. But from initial results it can be concluded that fresh embryos give better pregnancy rates than slow freezed and vitrified embryos. Still many more studies on indigenous breeds are needed to improve their population in India.

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