Effect of cryopreservation of *in vitro* produced embryos on pregnancy rate of cows transferred at fixed time in the dry tropics

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ABSTRACT
The objective of this study was to determine the effect of cryopreservation (CP) of *in vitro* produced embryos (EIV) on the pregnancy rate (PR) of cows transferred at fixed time (FTET) in the dry tropics. The experimental design was completely randomized with a 2×2 factorial arrangement. The sample consisted of 280 embryos produced *in vitro*. The factors were the CP (vitrified and fresh) and the Rural Development District (DDR 01 and 02). The experiment consisted of 70 replicates per treatment: T1) fresh transferred embryos; T2) vitrified embryos; T3) DDR + fresh embryos; and 4) DDR + vitrified embryos. The pregnancy diagnosis was carried out 60 days after embryo transfer. The Xi^2 test was used to analyze PR which was the response variable per treatment. PR was higher in fresh-transferred embryos than in vitrified embryos (53.6±4.2 *vs.* 27.1±3.7; $P<0.05$), and in DDR02 than in DDR01 (47.1±4.2 *vs.* 33.6±3.9; $P<0.05$). Therefore, the transfer of fresh embryos and the DDR02 had a positive overall effect on PR (40.3%).

Keywords: Biotechnologies, Synchronization, Ovulation, Cows.

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INTRODUCTION

The embryo transfer (ET) requires selection and physical and pharmacological handling of the receptor cows; the success of this biotechnological procedure depends on many factors [1]. *In vitro* fertilization and cryopreservation (CP) of embryos help to increase their mass production, their storing in germplasm banks, and their transportation around the world [2]; the ultimate aim of the producers is to use embryos in transfer programs. The in vitro production (IVP) of embryos is an assisted reproduction biological tool that speeds up the genetic progress of animals. This tool allows a genetically superior female to produce a greater number of embryos (and consequently offspring) during her reproductive life. Under natural mating or artificial insemination systems, a cow would give birth to only one offspring per year [3].

Non-transferred fresh embryos subjected to CP can remain available for an undetermined time, before they are used for transfer or for commercial purposes [4]. Vitrification (VT) is one of the methods used to cryopreserve cattle embryos. This method allows the change from liquid to solid without the formation of intracellular ice crystals [5]. VT is an alternative for embryo preservation with optimal viability percentage. Additionally, this is a simple, easily executed, cost-efficient technique, that results in 40-46% pregnancy rates for in vitro produced embryos transferred vitrified or fresh [6, 7]. In 2022, the International Embryo Technology Society (IETS) reported that 1,521,018 *in vitro* produced embryos were transferred all over the world in 2021. Out of this total, 32.6% were cryopreserved embryos, while 67.4% were fresh-transferred embryos. South America is the world leader in this biotechnology, with 378,114 transfers of fresh embryos and 279,291 transfers of frozen embryos. A total of 91,954 *in vitro* produced embryos derived from follicular aspiration was reported in Mexico; out of this total, 8.2% were transferred fresh, which accounted for a production increase of 244.6% compared with 2020 [8]. Therefore, the objective of this study was to determine the effect of cryopreservation of *in vitro* produced embryos on pregnancy rate of cows transferred at fixed time in the dry tropic.

MATERIALS AND METHODS

This study complied with the requirements of the Comission of Bioethics and Animal Welfare and the guidelines of the Internal Rules of Procedure of the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Veracruzana (Title VII, Chapters I, II, III, IV, Articles 92-124) and the NOM-033-ZOO-1995, clause 6.1.b.

Lab location

The *in vitro* production of embryos was carried out in the Laboratorio RGA IN VITRO (Reproducción Genética Avanzada), located in Boca del Río, Veracruz.

Location of the livestock production units

The cows from which the oocytes were extracted —and which were used for the *in vitro* production of embryos— belonged to a commercial dairy herd (Rancho Fuentezuelas) located in Tequisquiapan, municipality of San Juan del Río, Queretaro (20° 31' N and 99° 53' W, at 1,870 m.a.s.l.). The transfer of the *in vitro* produced embryos was carried out in dual-purpose cattle ranches, located in the municipalities of Atoyac de Álvarez and Florencio Villareal, which belong to Rural Development Districts 01 and 02, respectively. These RDDs are located in the State of Guerrero. The municipality of Atoyac de Álvarez is located in the Costa Grande of Guerrero (17° 03' N and 100° 05' W, at 602 m.a.s.l). The municipality of Florencio Villarreal is located in the Costa Chica of Guerrero (16° 43' N and 99° 07' W and at 16 m.a.s.l).

Characteristics and management of the donors

Twenty non-lactating and non-pregnant Holstein cows were used. They were included in a multiple ovulation commercial program and kept in pens. All the cows were fed

conserved forage and a balanced diet prepared at the farm with 16% CP (3.2 Mcal/kg) and mineral salts. Cows had *ad libitum* access to water. Based on the transrectal ultrasound carried out during the examination of the selected cows, their reproductive tracts showed no anomalies [9]. Body condition (BC) of the cows was 3.4±0.1 points (1 to 5 points scale: 1=extremely thin and 5=obese) [10]. The cows were apparently healthy and they complied with the local deworming and vaccination plans.

Characteristics and management of the recipients

Two-hundred eighty *Bos indicus*×*Bos taurus* recipient cows were used in the experiment. They were 4.5±1.2 years old and weighed 482.5±69.2 kg (live weight). At least 60 days had elapsed since parturition. The cows were apparently healthy. In order to discard pathological or anatomo-functional alterations, a transrectal palpation of their reproductive tract was carried out. Additionally, the selected recipients met the following requirements: 1) BC from 5 to 7 in the 1-9 dual-purpose cattle scoring (1=extremely thin and 9=obese) [11]; 2) not being under any pharmacological treatment; and 3) showing signs of reproductive activity, whether by the presence of a corpus luteum (CL) detected during transrectal palpation or by evidence of follicular activity detected through transrectal ultrasound of the ovaries (Aloka SSD500 portable ultrasound, with a 5.0 MHz transrectal transducer probe) [9, 12], following the recommendation of the IETS.

The feeding of the recipients was based on semi-extensive rotational grazing on Gamba grass (*Andropogon gayanus* Kunth), Mulato grass (CIAT 36061), and giant star grass (*Cynodon plectostachium*). In addition, they were supplemented with 2 kg cow^{-1} d^{-1} of a commercial concentrate feed with 19% CP (Lechero 20 CSA Malta®, Texo de México S.A de C.V) 30 days before and 30 days after the embryo transfer. They were also given *ad libitum* a mineral supplement with a higher bioavailability of phosphorus.

Health management included a 5-mL subcutaneous (SC) application of a bacterin-toxoid (Bobact 8® MSD, Mexico), in order to prevent pneumonic pasteurellosis (shipping fever), blackleg, malignant edema, gas gangrene, necrotic infectious hepatitis, and enterotoxaemia (pulpy kidney). In addition, they were vaccinated against IBR, BVD (types 1 and 2), Pl3, and BRSV; this vaccine includes the *Campylobacter fetus* bacterin, combined with inactive *Leptospira* (2 mL/SC, Bovi-Shield GOLD® FP® 5 VL5. Zoetis, Mexico). The cows were dewormed with 1% doramectin (200 mcg/kg PV, SC, Dectomax® Zoetis, Mexico) to prevent lungworms and gastrointestinal parasites. They were given a bath every 15 days to prevent ticks, using 12.5% amitraz (Taktic®, MSD, México). Seven days before the start of the ovulation synchronization protocol, each recipient received via intramuscular (IM) 10 mL of phosphorus (Tonofosfán®, MSD, Mexico), 8 mL of selenium (Mu-Se®, MSD, Mexico), and 5 mL of vitamin A, D, and E (Vigantol®, Bayer, Mexico).

Ovulation synchronization program

On Day 0, ovulation was synchronized in all the recipients using a progesterone-releasing intravaginal device (CIDRB®; Zoetis, Mexico) together with the IM injection of 2 mg of estradiol benzoate (Benzoato de estradiol®; Zoetis, Mexico). On Day 5, cows received via IM 400 IU of equine chorionic gonadotropin (eCG; Novormon®; Zoetis, Mexico) and 25

mg of dinoprost tromethamine (Lutalyse; Zoetis, Mexico). On Day 8, the CIDR-B was removed and the recipients received via IM 1 mg of estradiol cypionate (E.C.P.®, Zoetis, Mexico) [13].

Characteristics of the transferred bovine embryos

Two-hundred eighty bovine embryos produced in vitro (140 fresh embryos and 140 embryos vitrified in Cryotop® devices) were used in the experiment. They were quality 1 compacted blastocysts. The ovum pick-up (OPU) technique was used to obtain the oocytes which originated the embryos; they came from Holstein donors that were subjected to in vitro fertilization with semen of registered Gyr bulls (JCVL215; Astro FIV Cabo Verde).

***In vitro* embryo production technique**

Preparation of the donors and follicular aspiration (OPU)

Before each procedure, the feces were removed from the rectum of the donors and the perineal region was washed with water and 70% ethanol. Epidural anesthesia (100 mg lidocaine; Lidocaína®, Lab. Intervet, Mexico) was applied before each OPU session, in order to reduce intestinal peristalsis and the pain from the procedure. The transducer was inserted via transvaginal and the ovary was paired with it through transrectal manipulation to conduct the aspiration of all the visible follicles. Follicular aspiration was performed by a single trained technician using a B-mode real-time ultrasound scanner (Mindray 2200®; Mindray Bio-Medical Electronics, Shenzhen, China) equipped with a 5 MHz micro-convex traducer (Mindray 65C15EAV®, Mindray Bio-Medical Electronics, Shenzhen, China) connected to a follicular aspiration probe (Watanabe Tecnologia Aplicada®, São Paulo, Brazil) and a stainless-steel needle. Follicular puncture was carried out using an 18 G disposable hypodermic needle (Jelco®, Fibra Cirúgica, Santa Catarina, Brazil) connected to a 50-mL conical tube (Corning®, Acton, MA, USA) with a silicone tube (Watanabe Tecnología Aplicada®, São Paulo, Brazil). A vacuum pump (WTA model BV-003®, Watanabe Tecnología Aplicada, São Paulo, Brazil) with negative pressure adjusted to 60-80 mmHg was used to keep the pressure level during the aspiration. After the OPU was carried out in both ovaries, the aspiration system was washed with DPBS medium (Nutricell®, Nutrientes Celulares, São Paulo, Brazil), 0.05% sodium heparin (5,000 UI/mL, Hemofol®, Crist alia Produtos Químicos Farmacéuticos, São Paulo, Brazil), and 1% fetal bovine serum (Gibco®, Thermo Fisher Scientific, MA, USA). Immediately after the OPU session, the oocytes were examined and classified according to their morphology (number of layers, expansion of the cumulus cells, and cytoplasm appearance regarding color, homogeneity, and integrity) [14].

***In vitro* embryo production**

The IVF procedures were adapted from Pontes *et al.* [15].

***In vitro* maturation**

The oocytes were cultured in 20-μL drops of BO-IVM maturation medium (IVF Bioscience®, Cornwall, UK), previously warmed. Five oocytes were used per drop,

under a mineral oil layer. They were matured in an incubator, with 5% CO_2, at 38.5 °C for 24 h.

Sperm preparation and in vitro fertilization

The oocytes were removed from the culture medium; they were washed and placed in 20 μL drops of previously warmed and gasified fertilization medium (five oocytes per drop), which were covered with a mineral oil layer. Frozen semen from the same Gyr bull was used to fertilize all the oocytes. The semen straws were thawed at 37 °C for 30 s; a sample of the semen was taken to verify its viability and motility. The semen was centrifuged in 45% Percoll gradient at 700G during 3 minutes and, subsequently, it was centrifuged in BO-SemenPrep (IVF Bioscience®, Cornwall, UK) medium at 700G during 1 minute. The spermatozoa were introduced in the drops of the fertilization medium that contained the oocytes. Both gametes were incubated at 38.5 °C, using 5% CO_2, for 18 h.

Maturation evaluation

The oocytes were removed from the fertilization medium and washed with HTF medium added with hyaluronidase (HTF/0.5 % BSA®, Sigma Aldrich, Saint Louis, USA) for 3 minutes; subsequently, they were denuded using a Stripper® (Origio; Ballerup, Denmark) device and were observed under a stereoscopic microscope. The presence of a polar body indicated an appropriate maturation.

***In vitro* culture**

Twenty-four hours after fertilization, the potential zygotes were cultured *in vitro*, in synthetic oviductal fluid (SOF) medium, supplemented with 5% fetal bovine serum and 0.5% bovine serum albumin. The cells were cultured for 24 hours in an incubator (Cook Minc™, USA), with a controlled gas atmosphere (5% CO_2 and 5% O_2, balanced with 90% N_2). On Day 4 (Day 0=IVF), the culture medium was renewed. On Day 6 (60 h after fertilization), the blastocyst rate was evaluated. The blastocysts were introduced into 0.25 cc straws and transported at 37 °C in an embryo transporter (TE-100 Compact WTA®, Watanabe Tecnología Aplicada, São Paulo, Brazil) for a maximum period of 12 h, until the moment of the transfer.

Vitrification technique

An equilibrium solution (ES) and a vitrification solution (VS) were used to vitrify the embryos. The ES was made up of phosphate buffered saline (PBS) (Fisher BioReagents®, Thermo Fisher Scientific Inc., MA, USA), 20% synthetic serum substitute (SSS) (Irvine Scientific® Santa Ana, CA, USA), 7.5% ethylene glycol (EG) (Sigma-Aldrich®, Darmstadt, Alemania), and 7.5% dimethyl sulphoxide (DMSO) (Sigma-Aldrich®, Darmstadt, Germany). The VS was composed by PBS + 20% SSS + 15% EG + 15% DMSO + 0.5 M sucrose (Sigma-Aldrich®, Darmstadt, Germany). One drop of ES and four drops of VS, 20-μL each, were placed in a 35×10 mm Petri dish (Nunc®, Thermo Fisher Scientific Inc., MA, USA). Afterwards, the embryos were introduced into the ES

drop for 5-15 minutes; subsequently, the embryos were transferred into each of the four VS drops, where they remained for 5, 5, 10, and 10 seconds. Immediately after they were removed from the last drop (<30 seconds in total), they were placed into Cryotop® devices, which were introduced in a cryogenic tank with N_2L where they were stored until the transfer [16].

Warming of the vitrified blastocysts

The vitrified embryos were warmed before their transfer. Solutions for thawing (TS), dilution (DS) and washing (WS) were used in this process. The TS was made up of PBS (Bioniche®, Pharma, Canada), 20% SSS, and 1 M sucrose (Sigma Aldrich®). The DS was composed by PBS + 20% SSS and 0.5 M sucrose). The WS was made up of PBS + 20% SSS. A 300 μL TS drop was placed in a 60×15 mm Petri dish, at 37 °C. Meanwhile, two DS drops and three 20 μL WS drops were placed in another 100×15 mm Petri dish, at room temperature. In order to warm the embryos, the Cryotop® was removed from the cryogenic tank, opened and immediately immersed in the TS drop for 1 minute. Subsequently, the blastocysts were placed in the DS drops for 3 minutes and after that in the WS drops, remaining 3 minutes in each one [16]. Finally, the embryos were placed in PBS and introduced into 0.25 cc straws to be transferred to the recipients.

Embryo transfer and pregnancy diagnosis

The embryo transfer was carried out by the same veterinarian in all the recipients that had a well implanted corpus luteum (CL) ≥1.5 cm in diameter 9 days after the removal of the intravaginal progesterone device [17]. To carry out the transfer, the recipients were given epidural anesthesia (100 mg lidocaine; Lidocaína®, Lab. Intervet, Mexico) 10 minutes before the procedure took place. In addition, iodine and 70% ethanol (Dermodine Solución™, Degasa, Mexico) were used to carry out a peri-vulvar asepsis. The embryo transfer gun was wrapped with a sterile cover sheath. It was introduced in the vagina and passed through the cervical channel (through transrectal manipulation) and was directed towards the uterine horn ipsilateral to the ovary with the corpus luteum, depositing the content of the straw in the middle third of the horn [18]. Each recipient received one embryo. Pregnancy diagnosis was carried out using a transrectal ultrasound (Aloka SSD500, with 3.5 MHz convex transducer; Japan), 60 days after the transfer.

Experimental design

The experimental design was completely random, with a 2×2 factorial arrangement. The sample size was 280 *in vitro*-produced embryos. The factors were CP and RDD, with 70 replicates per treatment.

Statistical analysis

A threshold mixed model was used with the PROC GLIMMIX procedure (SAS, 2014) to determine pregnancy rate (PR). Based on the fixed and random effects, the PR was assumed to follow a Bernoulli distribution. It was analyzed with the χ^2 ($p \leq 0.05$), using the STATISTICA™ version 10 software (TIBCO Software Inc., StatSoft, 2011, USA).

Statistical model

The following statistical model was used:

$$Y_{ijk} = \mu + B_i + O_j + BO_{ij} + \beta_1\left(x1_{ij} - x^{-1}\right) + \beta_2\left(x2_{ij} - x^{-2}\right) + \beta_3\left(x1_{ij} - x^{-3}\right) + \varepsilon_{ijk}$$

Y_{ijk}=response variable (pregnancy rate); μ=general mean of the response variable; B_j=*i*-th effect of the type of embryo, i=1,2; O_j= *j*-th effect of the RDD, j=1,2; BO_{ij}=effect of the interaction between the *i*-th effect of the type of embryo and the *j*-th effect of the RDD.

$\beta_1\left(x1_{ij} - x^{-1}\right)$= effect of the covariate age; $\beta_2\left(x2_{ij} - x^{-2}\right)$= effect of the covariate weight; $\beta_3\left(x1_{ij} - x^{-3}\right)$= effect of the covariate body condition; ε_{ijk}=experimental error.

where μ=general mean; B_i=*i*-th effect of type of embryo; O_j=*j*-th effect of the RDD; BO_{ij}=double of the interaction of the factors; ε_{ij}=experimental error.

RESULTS AND DISCUSSION

The PR of the fresh and vitrified embryos reached 44.3 and 22.8%, respectively, in RDD 01, and 62.8% and 31.4%, respectively, in RDD 02 (Table 1). The results were acceptable for the *Bos taurus*×*Bos indicus* recipients in the dry tropics. Pregnancy rate is a multifactorially-regulated event. The main factors that can have a negative impact on PR include: embryo development [19], embryo quality [20], the selection of the recipients [21], and the CL diameter (>14 mm) [17]. In all the treatments of this study, only embryos in the blastocyst development stage (early, blastocyst, and expanded) were transferred. Therefore, this variable did not likely have an impact on the pregnancy results. A research carried out in the sub-humid tropics showed that there were no differences in the transfer of fresh blastocysts (48%) or expanded blastocysts (52%) in Brown Swiss heifers [22]. Other authors have reported a similar PR when the transfers were carried out with early (30%), expanded (40%), and hatched blastocysts (50%), produced *in vitro* and vitrified using the Cryotop® device. These similarities could be the result of a synchronization between the embryo development and the uterine environment of the recipient (±24 h) [23], influenced

Table 1. Transferred and pregnant cows, and pregnancy rate post embryo transfer in production units (PU) of the Rural Development Districts (RDD) 01 and 02 in the state of Guerrero.

RDD / Type of embryo	Transferred n	Pregnant n	Pregnancy rate (%)
Fresh	70	31	44.3[b]
01			
Vitrified	70	16	22.8[bc]
Fresh	70	44	62.8[a]
02			
Vitrified	70	22	31.4[b]
Total	280	113	40.3

[a,b,c] Different letters indicate statistical difference (P<0.05).

by the progesterone (P4) from the CL. The P4 plays an important role in reproductive events, mainly in the establishment and maintenance of pregnancy [24]. In addition, there is a positive association between the high concentrations of blood P4 after conception and the elongation of embryos, the increase of interferon tau (IFN-t) production, and the high pregnancy rates resulting from *in vitro* produced embryos [25, 26]. In this study, the P4 plasma concentrations were not determined when the ET was carried out. However, with the aim of providing a sufficient P4 contribution during the next embryo stages, only cows with a CL >14-mm in diameter were included. In synchronized *Bos taurus*×*Bos indicus* recipients there was a positive correlation (r=0.41) between CL size (>14 mm) and the P4 plasma levels, resulting in a greater contribution of P4 for pregnancy maintenance [27]. According to Bó *et al.* [17], there are no differences in PR of recipients with CL of diameters of 14-16 mm (56.1%), 16-18 mm (56.4%), and >18 mm (54.3%).

The quality of the embryo is a main factor to consider when performing ET, as it has the greatest effect on the embryo development since the implantation [20]. Bad quality embryos impact PR, given the poor compaction of their blastomeres, low internal cell mass, presence of vesicles inside the cytoplasm, and scarce uniformity of the pellucid zone [28]. All these factors affect the embryo competence during [29]. Some authors mentioned that there is no difference when quality 1 (excellent) and quality 2 (good) embryos are transferred, due to their appropriate morphological and developmental characteristics according to their age, which are ideal for ET [30, 23]. Only quality-1 fresh and warmed vitrified-embryos were used in this study. They were evaluated before ET was carried out. This factor could have contributed to the appropriate embryo development and, therefore, the acceptable general pregnancy rates recorded per treatment.

Regardless of the type of embryo, PR was higher in RDD 02 than in RDD 01 ($p<0.05$) (Table 2). Irrespective of the RDD, PR recorded the highest percentages with fresh transferred embryos than with vitrified transferred embryos ($p<0.05$) (Table 2). The cattle producers of both RDD had similar livestock, management, infrastructure, and technological level, and they were given the same training and instructions, regarding the implementation of ovulation synchronization for FTET. However, RDD 02 recorded higher PR. Severino *et al.* [32] mentioned that the different technological levels in cattle production in Mexico can make a difference regarding the implementation of a biotechnology. This was the case in this study.

Table 2. Pregnancy rate of *Bos taurus*×*Bos indicus* recipients, considering the main effects RDD (01 and 02) and type of embryo (fresh vs vitrified) in the state of Guerrero, Mexico.

Main effects		**Pregnancy rate mean±SE % (pregnant/transferred)**
RDD	01	33.6±3.9 (47/140)[a]
	02	47.1±4.2 (66/140)[b]
Type of embryo	Fresh	53.6±4.2 (75/140)[a]
	Vitrified	27.1±3.7 (38/140)[b]

[a,b] Different letter indicates statistical difference ($p<0.05$).

Meanwhile, the PR obtained with vitrified embryos was acceptable, although it was slightly lower than that obtained with fresh embryos. The latter was higher than the PR obtained by Gutnisky *et al.* [6] with *in vitro* produced embryos transferred fresh and vitrified (46% for both treatments), and by Do *et al.* [7], who reported 41% and 34% PR with fresh and vitrified *in vitro* produced embryos, respectively. Post-warming *in vitro* viability of embryos vitrified in Cryotop® and Microdrop® devices can reach 70-90% [32, 7], resulting in appropriate embryo development that is reflected in acceptable PR for both *in vivo* and *in vitro* produced embryos (46.7 *vs.* 44.4 %) [33].

Transferring vitrified embryos can result in a 10% lower PR than transferring fresh embryos [30, 34]. Nevertheless, the PR obtained in this study in *Bos taurus*×*Bos indicus* recipients with vitrified embryos can be considered acceptable and with practical application in the field. Vitrification can prevent the discarding of excellent quality embryos, and help make better use of the number of available recipients. Therefore, this method is a feasible alternative for the genetic improvement of commercial cattle herds, under tropical conditions. Nevertheless, embryo vitrification is still a technology with a low percentage of use in the Mexican tropics.

CONCLUSIONS

The fixed time-embryo transfer of fresh and vitrified, *in vitro* produced Girolando embryos, to dual-purpose cows owned by small producers in the dry tropics, resulted in acceptable pregnancy rates. Consequently, this is an alternative for the genetic improvement of such cattle herds.

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