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Article

Effect of vitrification on the *in vitro* and *in vivo* development of bovine embryos and hemi-embryos produced *in vivo*

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Abstract:

The effect of bipartition on the *in vivo* and *in vitro* development of bovine embryos and hemi-embryos produced *in vivo* after vitrification was evaluated. In experiment 1, 90 vitrified bovine embryos were used, which were distributed in two treatments: EC= embryo culture (n= 21), EC1: culture of 11 embryos (22 hemi-embryos), and EC2: culture of 10 whole embryos. The development of hemi-embryos and whole embryos was evaluated after warming every 24 h to 72 h. Experiment 2 assessed the pregnancy rate (PR; n= 69). It was divided into 2 groups, PR1= transfer of 31 bipartite embryos (62 hemi-embryos), and PR2: transfer of 38 whole embryos. For the viability analysis of bipartite *versus* whole embryos at 24, 48, and 72 h, the model included only the treatment effect, using the probit function, and for the pregnancy rate analysis, a mixed threshold model was used with SAS PROC GLMMIX. In embryo culture, a final embryonic development of 77.3 % was observed for EC1 *vs* 80 % in EC2 (P>0.05). The pregnancy rate was 19.35 *vs* 31.6 % in PR1 and PR2,

respectively (P>0.05). It is concluded that, under the conditions of the present work, the bipartition of vitrified and warmed embryos did not affect the *in vitro* or *in vivo* development of bovine embryos and hemi-embryos produced *in vivo*. In addition, with bipartition, the same number of pregnancies was obtained with fewer embryos.

Keywords: Bipartition, Blastocyst, Vitrification, Embryonic development.

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Introduction

Since the 1980s, reproductive biotechnologies in cattle have evolved efficiently through the development of various techniques, which has helped to rapidly increase reproductive capacity and increase the economic cost of each calf born[(1)]. The techniques that have been most widely applied are embryo transfer (ET), embryo cryopreservation, and embryo manipulation (bipartition) in order to obtain more animals with greater productivity potential[(2)].

The bipartition of bovine embryos is a very useful technique for generating homozygous twins and an important advantage is that of obtaining greater utilization of embryos of high genetic value[(2,3)]; another advantage of this technique is to increase the number of opportunities that an embryo has to implant and thus have a better use of the genetic material and the recipient females[(4)]. Pregnancy rates for fresh bipartite and fresh transferred bovine embryos range from 55 to 61 %, and are similar to the pregnancy rate for embryos transferred complete, which is 50 to 60 %[(5)].

Embryo transfer is a technique that is implemented in genetic progress programs that seek to obtain a bigger number of offspring from donor females with a high productive potential[(6)]; in addition, these programs are complemented by the cryopreservation of embryos that are not transferred fresh or embryos that are destined for marketing and storage for subsequent transfer[(7)].

One of the methods of embryo cryopreservation is the vitrification technique, which allows the transition from the liquid to the solid state without the formation of intracellular ice crystals[(8)]. Vitrification is an optional technique for preserving embryos with optimal viability percentages and is also a simple, easy-to-perform and cost-effective technique[(9)].

Pregnancy rates after transfer of warmed (devitrified) bovine embryos range from 45 to 65 %[10,11,12].

In the Mexican tropics, the predominant cattle production system is the so-called dual-purpose one, where there are commonly high-value embryo donor cows. Based on the above and given the scarcity of recent information on this subject, this study aimed to evaluate the effect of the bipartition of vitrified and warmed bovine embryos on *in vivo* and *in vitro* development; if there is no negative effect, this would allow to increase the number of offspring obtained per embryo and make optimal use of them.

Material and methods

Embryo production and treatments

A bank of 90 *Bos taurus* x *Bos indicus* blastocysts of quality 1 (according to the IETS classification), produced *in vivo*, cryopreserved by vitrification in Cryotop® (Kitazato Supply, Fujinomiya, Japan)[13] was used.

In vitro development was evaluated by embryo culture (EC) for 72 h after warming. *In vivo* development assessment was performed by determining the pregnancy rate after embryo transfer (PR). Each experiment was divided into two groups: EC1= culture of 11 bipartite embryos (22 hemi-embryos) and EC2= culture of 10 whole embryos; PR1= gestation rate of recipients transferred with hemi-embryos (n= 62) and PR2= pregnancy rate of recipients transferred with whole embryos (n= 38).

Embryo vitrification

The embryos were vitrified as previously described[14]. The embryos and hemi-embryos were placed in a 25 µL drop of equilibrium solution (ES; PBS with 7.5 % v/v ethylene glycol and 7.5 % v/v dimethyl sulfoxide) for 10 min. After this, the embryos and hemi-embryos were passed through 4 drops of 25 ul in a vitrification solution (VS; PBS with 15 % v/v ethylene glycol and 15 % v/v dimethyl sulfoxide) for one minute in total, that is, from the time they were placed in the first drop to the fourth drop. Immediately afterwards, the embryos and hemi-embryos were placed in the cryotop device in the lowest possible volume (approximately 1 to 2 uL) and immersed in liquid nitrogen.

Embryo warming

For this, a warming solution (WS) [phosphate buffer solution (PBS) with 20 % synthetic serum substitute (SSS) and 1 M sucrose], a dilution solution (DS) (PBS with SSS at 20 %

and 0.5 M sucrose), and a washing solution (WS) (PBS with 20 % SSS) were used. The Cryotop® was removed from the liquid nitrogen, uncapped and immersed in the WS for 1 min, in the DS for 2 min in each 25 uL drop, and in the WS for 3 min in each 25 uL drop[14]. The embryos were then placed in a culture medium and the embryos to be transferred in PBS and placed in 0.25 mL straws for transfer to the recipients.

Embryo bipartition

After warming and before being introduced for embryo culture or transfer, 53 embryos were bipartitioned using the modified technique described by Lopes *et al*[5], which consists of placing the embryo in a disposable Petri dish (100 x 15 mm; Corning®, New York, USA) with micro manipulation medium (DPBS, Dulbecco's Phosphate-Buffered Saline). The division was carried out with a micromanipulator (MWS-1A, Narishige, Tokyo, Japan) placed in an inverted microscope (CK2, Olympus®, Tokyo, Japan), to which a microblade was adapted, making the cut in such a way as to divide the internal cell mass into equal parts. After bipartitioning, the hemi-embryos were placed in culture medium or loaded into 0.25 ml straws to be transferred to the recipient females.

In vitro embryonic development assessment

Embryo culture

A total of 21 warmed embryos were used, of which 11 were bipartite, resulting in 22 hemi-embryos (EC1) and the remaining 10 were cultured complete (EC2). All hemi-embryos and embryos were cultured in IVC3 medium (*In Vitro* Care, Maryland, USA) in an incubator at 38.5 ºC, 5 % of CO_2 in air and humidity of 95 %. These were reviewed every 24 h for 72 h to evaluate the development of the multiplication of blastomeres (re-expansion) and of the whole embryos until hatching[15].

In vivo embryonic development assessment

Embryo transfer

The Bioethics and Animal Welfare Commission of the FMVZ-UV approved the use of animals and the procedures included in the study. For embryo transfer (n= 69), 100 *Bos taurus* x *Bos indicus* recipient females were selected, with an average age of 60 ± 2.71 mo, 2.26 ± 1.04 births, 454 ± 6.09 kg of weight, body condition between 2.5 and 3.0 (5-point scale: 1= emaciated and 5= obese)[16] and clinically healthy. The recipients were evaluated by transrectal palpation to corroborate their cyclic status. Recipients that were cycling were used, that is, recipients that had a corpus luteum in one of the two ovaries and that no

pathologies were detected in the reproductive tract. The recipients were under rotational grazing of Pangola grass (*Digitaria eriantha*) and Insurgente grass (*Brachiaria brizantha*), received mineral salts and water *ad libitum*, and each received 4 kg of concentrated feed per day (the feed contained 20 % crude protein and was administered 30 d before and 30 d after the ET). On day -60 (day 30= start of ET treatment), recipients received the following intramuscularly: vitamins A, D and E 1 (1 250 000, 35 0000, and 350 IU, respectively; Synt-ADE®, Zoetis, Mexico), selenium plus vitamin E (40 and 400 mg, respectively; Selenie®, Virbac, Mexico), and phosphorus (2 g; Phospho 20®, Virbac, Mexico).

Synchronization of ovulation of recipients

For embryo transfer, the recipients were divided into two treatments, PR1: 62 (hemi-embryos) and PR2: 38 (whole embryos). On day 0, recipients received an intravaginal device containing 1.9 g of progesterone (CIDR®, Zoetis, Mexico) and 2.5 mg of estradiol benzoate i.m. (Estradiol Benzoate®, Zoetis, Mexico). On day 5, 400 IU of equine chorionic gonadotropin eCG i.m. (Novormon®, Zoetis, Mexico) and 25 mg of dinoprost tromethamine i.m. (Lutalyse®, Zoetis, Mexico) were applied. On day 8, the CIDR-B® was removed and 1 mg of estradiol cypionate i.m. (E.C.P.®, Zoetis, Mexico) was applied[17].

Non-surgical embryo transfer

Embryo transfer was only performed in those females that had a quality 1 corpus luteum (20 mm in diameter), detected by ultrasound. The embryo transfer was performed 9 days after removing the CIDR-B®; epidural anesthesia was administered 5 min before the transfer (100 mg, Lidocaine®, Lab MSD). The whole embryos or hemi-embryos were placed in the ipsilateral uterine horn of the corpus luteum[18]. Each of the recipients received either a hemi-embryo (n= 62) or a whole embryo (n= 38).

Pregnancy diagnosis

On d 60 after the transfer, the diagnosis of pregnancy was made by ultrasound. This waiting time is necessary since there is information that, if performed before 60 d of pregnancy, the main period of organogenesis, it constitutes an important iatrogenic cause of irreversible damage or fetal loss[19].

Statistical analysis

A mixed threshold model[20,21,22] with PROC GLIMMIX (SAS, 2014) was used for the analysis of the pregnancy rate. Conditioned to fixed and random effects, it was assumed that the pregnancy rate followed a Bernoulli distribution. To analyze the pregnancy rate and

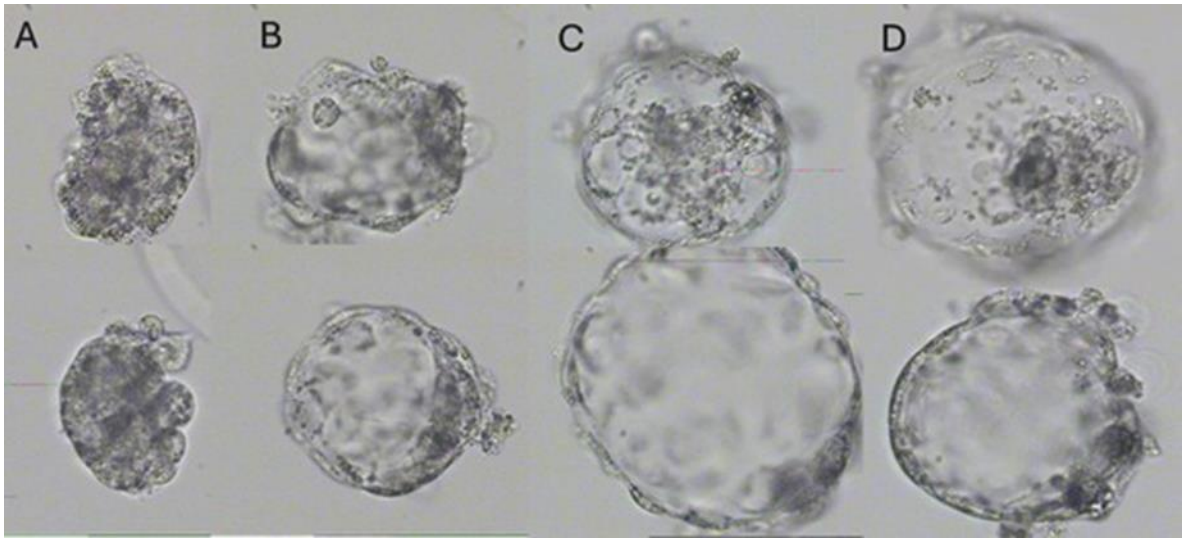
viability of hemi-embryos *versus* that of whole embryos at 24, 48 and 72 h, the model included only the treatment effect. The linking function used was the probit function. The solutions for random fixed effects were based on maximum likelihood techniques using the average information matrix algorithm to estimate the variance components. To calculate the degrees of freedom, the Satterthwaite approach was used. Comparisons between the effects of the means considered in the model were made based on Fisher's least significant difference.

Results

In vitro viability of hemi-embryos and whole embryos

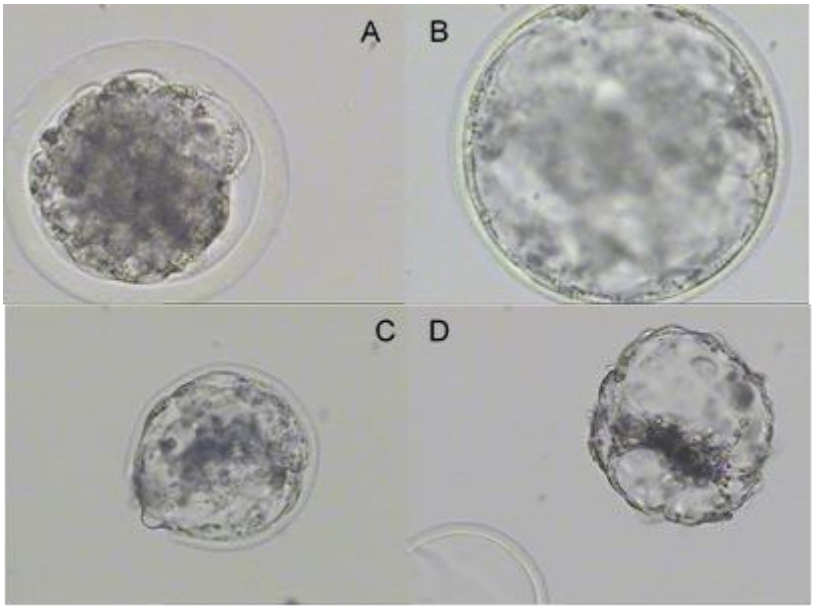
For the EC1 treatment, there was a viability of 86.3 % (19/22) at 24 h, decreasing to 81.8 % (18/22) at 48 h and finally to 77.3 % (17/22) at 72 h after blastomere multiplication (re-expansion; Figure 1).

Figure 1: Hemi-embryo culture at 72 h A) Hemi-embryos at 0 h of culture, B) Re-expansion of hemi-embryos at 24 h, C) Re-expansion of hemi-embryos at 48 h, D) Re-expansion of hemi-embryos at 72 h



For the EC2 treatment, the cultured whole embryos had a viability of 90 % (9/10) at the first 24-h review, the same expansion rate (90 %) (9/10) was obtained at 48 h, but at 72 h, the hatching percentage reached 80 % (8/10; Figure 2).

Figure 2: Culture of whole embryos: A) Newly warmed embryo (0h), B) Embryo cultured at 24 h, C) Embryo cultured at 48 h, D) Embryo cultured at 72 h



When analyzing the embryo viability of both groups, no statistical difference (P>0.05) was found in the different evaluation times (Table 1).

Table 1: Viability rate at 24, 48 and 72 h after warming of bipartite and whole embryos

Treatment (n)	Viability time of evaluation (%)		
	24	48	72
EC1: Bipartite (22)	86.3	81.8	77.3
EC2: Whole (10)	90	90	80

(P>0.05).

In vivo viability of hemi-embryos and whole embryos

Gestational rate with embryos produced *in vivo*, vitrified, bipartitioned and whole

For the PR1 treatment (n= 62 hemi-embryos), a pregnancy rate of 19.35 % (12/62) was obtained. The remaining transfers belonged to the PR2 treatment (n= 38) with whole embryos, with a 31.6 % (12/38) pregnancy rate (Table 2).

Table 2: Pregnancy rate of hemi-embryos and whole embryos

Embryos (n)	Transfers (n)	Pregnant (n)	Pregnancy rate by transfer (%)
PR1: Bipartite (31)	62	12	19.3
PR2: Whole (38)	38	12	31.6

(P>0.05).

Therefore, no statistical differences were found for both groups (P>0.05). Both the hemi-embryo group and the group of whole embryos obtained a total of 12 pregnancies, but with the difference that a smaller number of embryos were used in the hemi-embryo group.

Discussion

In the present study, the average viability rate of hemi-embryos and whole embryos was 78.65 % using IVC3 medium. There is little information about the development of bovine embryos that were produced *in vitro*, bipartitioned, vitrified and devitrified; therefore, the present work contributes to the knowledge about the viability of hemi-embryos and whole bovine embryos produced *in vivo*.

Based on the results of the present study in embryo culture, 86.3 % and 90 % viability were found for hemi-embryos and whole embryos 24 h after warming. This percentage is higher than that previously reported in rabbits[23]; in this study, they used 125 rabbit embryos produced *in vivo*, one group of embryos were vitrified and bipartitioned (as in the present work) and the other group were bipartitioned and then vitrified; the rate of *in vitro* development was 36 % in the vitrified/bipartitioned embryos and 10 % in the bipartitioned/vitrified embryos. These researchers mention that this could be due to the fact that the hemi-embryos lose cohesion and reduce the number of cells, and that bipartition leaves the cells exposed to the cryoprotectants used in vitrification, which can be toxic and affect embryonic development; therefore, they concluded that the quality of the embryo is the main factor in the success of these procedures. Other researchers mention that it has been proven that cells in the inner mass (about 10 %) become damaged due to the division procedure[24] and that an additional proportion of cells will die during freezing and thawing. In this regard, it is worth mentioning that, although staining was not performed in the present study to determine viability, the fact of obtaining a rate of 77.3 and 80 % viability *in vitro* 72 h post-warming, for hemi-embryos and whole embryos, shows that, at least under the conditions of the present work, the bipartition of vitrified/warmed embryos did not affect the *in vitro* development of hemi-embryos and whole embryos (P>0.05). It is important to

mention that, in the present study, only grade 1 embryos were used, while the aforementioned study used quality 1 and 2 embryos.

Cryopreservation reduces embryo division. Some studies have reported development rates of 52 % for frozen-thawed biopsied embryos compared to 60 % for fresh embryos. On the other hand, survival rates of 86 % have been published for vitrified and warmed embryos that had undergone assisted hatching[25].

The overall pregnancy rate (PR= 25.5 %) obtained in the present study with hemi-embryos and whole embryos in dual-purpose recipients was lower than that reported by other authors[26,27,28]; nevertheless, these variations obtained in PRs depend on several factors related on the one hand to the recipient and on the other hand to the embryo that is transferred[29,30,31]. The main factors that have been correlated with the success of pregnancy are embryo quality, cryopreservation method, synchronization protocol, and the technician who transfers it[12]; however, in the present study, only quality 1 blastocysts were used, their viability was evaluated after thawing, and the technician who performed the transfers was the same, so these factors probably did not affect the pregnancy outcomes.

Regarding the CIDR-B-based synchronization protocol of recipients, it has been reported that they influence PRs based on the quality of the corpus luteum formed and the environment that is fostered in the uterus after synchronization[31]. In this study, no difference was found in the size of the corpus luteum (≥20 mm) at the time of transfer in the PR; unfortunately, it was not possible to measure P4 in blood serum to reaffirm it; nevertheless, similar results were reported[32], finding no relationship between the diameter of the corpus luteum (>1.6 cm) and its function (plasma P4 >1 ng/mL) on d 6 of ovulation in heifers treated with CIDR-Bs and EB as basis of the protocol. In contrast[17], other researchers reported an increase in PRs in recipient heifers (*Bos indicus* crosses) due to a larger diameter of the corpus luteum at the time of embryo transfer, the recipients were classified according to the area of the corpus luteum as CL1 (> 2.0 cm^2), CL2 (1.5-2.0 cm^2), and CL3 (<1.5 cm^2), and the PRs obtained were 58.4, 41.5, and 31.8 %, respectively. These differences suggest that during the intermediate phase between the growth phase (up to d 7) and the static phase of the corpus luteum (d 8 to 16), luteal blood flow may be more indicative of corpus luteum function than its maximum size reached in PRs[33]. This possibility suggests that the luteal phase induced in the recipients of the present study was adequate in length, and what was surely compromised was the early development of the embryos, to the extent that they were not able to generate the adequate signal of early recognition of pregnancy (interferon-τ)[34,35,36]. In this sense, in the case of hemi-embryos, it should be considered that a factor that can affect the implantation of embryos is a failure during the signal sent by the embryo in the maternal recognition through the production of interferon-τ[37,38], hemi-embryos may have a lower production of interferon because they have fewer trophectoderm cells[38,39]. That is why it is

important to mention that, in order to perform maternal embryo recognition, the embryo must find an appropriate uterine environment, influenced by the progesterone produced by a good quality corpus luteum, since it stimulates the production of a variety of endometrial secretions, such as MUC-1 (mucin glycoprotein-1), placental lactogen, and osteopontin, necessary for the correct development of embryos[37]. In another study on embryo bipartition[38], they compared the pregnancy rate of recipients that were transferred with whole fresh embryos produced *in vivo* and hemi-embryos, obtaining a higher pregnancy rate with whole embryos (67 %) than with hemi-embryos (35 %) ($P<0.05$). These results are higher than those of the present study, where no statistical difference was found between whole embryos and hemi-embryos (31.6 *vs*. 19.3 %, respectively). One of the possible causes of the higher percentage is that they used Holstein heifers, which are a breed of *Bos taurus* origin that present greater fertility compared to the recipients that were used in the dual-purpose production system, *Bos taurus* vs *Bos indicus* crosses. In addition, another cause is that the embryos were transferred fresh and produced *in vivo* compared to this work, where the percentages were lower considering that the embryos went through a vitrification and warming treatment, and therefore, during the drop in temperature, damage to the embryo can be induced.

Recently[39], the effect of bipartition during embryo transfer was evaluated; blastocyst stage embryos were used, which were bipartitioned and transferred to the recipients. Fourteen days after the transfer, non-surgical embryo collection was performed and 37.5 % of elongated hemi-embryos were obtained *vs* 52.9 % of elongated whole embryos; these results are higher than those obtained in the present study, which were 19.35 *vs* 31.6 % for hemi-embryos and whole embryos. Nonetheless, the results could be considered favorable considering that they went through a cryopreservation process, and this could have a negative effect on achieving adequate implantation in the recipient. Although the elongation of the transferred embryos was not analyzed in the present study, it could be assumed that, since the embryos were elongated, it is very likely that they had implanted and produced pregnancies. Therefore, it was compared with the pregnancy rate obtained in the present study. In this sense, research on embryo cryopreservation[40] has mentioned that this is not a problem-free process since it induces extreme variations in the physical and chemical properties of the cell membrane, organelles, and the delicate cell-cell interaction inherent to the embryos to be cryopreserved. Likewise, the critical periods for embryonic survival during cryopreservation are the initial freezing phase and the period of return to physiological conditions. Cryo-induced embryonic lesions are explained by the formation of intracellular ice crystals and the osmotic stress to which the plasma membrane is subjected during freezing[41].

For a long time, there has been concern about whether calves born from hemi-embryos behave in the same way as those produced by whole embryos. A recent publication provided valuable information about this[42]; when comparing the development of embryos and hemi-

embryos from 60 d of pregnancy to 550 d of age, no differences were detected in the biparietal, abdominal, umbilical cord, orbital and aortic diameters. There were also no differences in birth weight, weaning weight, and the weight every 30 d up to 550 d. Therefore, they concluded that there are no physically discernible differences between embryo and hemi-embryo offspring, which is important for the safe use of the bipartition technique. This is important, especially because vitrification and bipartition are assisted reproduction techniques that have the potential to be used, not only in species of zootechnical interest, but also in endangered species[43].

Conclusions and implications

Under the conditions of the present work, the bipartition of bovine embryos produced *in vivo* does not affect *in vitro* development or pregnancy rate. With embryo bipartition, the same number of pregnancies was obtained with fewer embryos, providing recent information on these biotechnologies.

Acknowledgements and conflict of interest

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