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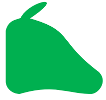
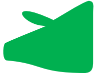
Technical note



Pregnancy and early pregnancy loss rates in cows receiving *in vitro*-produced vitrified embryos and hemi-embryos



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Abstract:

Bovine embryo bisection and vitrification protocols can affect pregnancy and early pregnancy loss rates. It was compared pregnancy rates at 30 d post-transfer and early pregnancy loss rates at 60 d post-transfer using *in vitro*-produced vitrified bovine embryos and hemi-embryos. Sixty (60), grade 1 bovine blastocysts were randomly distributed into two treatments: (1) Vitrified whole embryos (n= 30), and (2) vitrified bisected embryos (hemi-embryos) (n= 30). Embryo bisection was done prior to vitrification. Whole embryos and hemi-embryos were vitrified, stored, thawed, and transferred to 60 recipients. Pregnancy was diagnosed by transrectal ultrasound at 30 d post-transfer, and pregnancy loss measured at 60 d. Pregnancy and pregnancy loss rates were analyzed as binomial variables (0= not pregnant; 1= pregnant) and compared using Fisher's exact test. Neither rate differed between the hemi-embryos and whole embryos. Pre-vitrification bisection of *in vitro*-produced embryos affects neither pregnancy rate nor early pregnancy loss rate. This technique simplifies the transfer process because it removes the need for in-field bisection.

Key words: Bovines, Cryopreservation, Bisection.

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Production of bovine embryos by follicular aspiration and *in vitro* fertilization (IVF) are in increasing demand worldwide. In the last decade alone, it is estimated that more embryos have been produced by IVF than by conventional methods[1]. This growing preference is due to the fact that with IVF each semen dose (sexed or conventional) can be more efficiently used, which translates into more embryos per straw. This technique also allows extraction of oocytes from pregnant females, has high repeatability and is an alternative in cows that do not respond to conventional multiple ovulation treatments[2]. However, IVF embryo counts can vary widely in response cow breed, age and reproductive status, oocyte management, environmental conditions and semen quality. Moreover, there are reports of high genetic value cows that only produce one or two embryos per session in aspiration and IVF programs, a clear limitation with this technique[3,4].

In addition to the low embryo counts produced with this technique, embryo viability can be compromised when cryopreserved with the slow curve method. This occurs due to unfavorable characteristics such as a low blastomere count, mainly in the internal cell mass[5], and a large number of lipid droplets within the cytoplasm. Both increase embryo losses[6]. These challenges highlight the need to consider techniques such as embryonic bisection (division of an embryo into similar halves)[7], and embryo vitrification (ultra-rapid freezing in a glass-like state)[8]. These may improve pregnancy rates by increasing the probability that an embryo results in a pregnancy and reducing the damage caused by cryopreservation. The present study objective was to evaluate the effect of pre-vitrification bisection in *in vitro*-produced bovine embryos on pregnancy rates at 30 d and pregnancy loss rates at 60 d post transfer.

Embryos were produced from oocytes extracted from ovaries collected at a slaughterhouse (TIF 672). After collection, the ovaries were transported to the laboratory in plastic bags in a cooler kept at approximately 35 °C. In the laboratory, they were washed with saline solution and all follicles 2 to 8 mm diameters aspirated. Aspiration was done with a 10 ml syringe fitted with an 18 x 1 ½ gauge needle. The recovered liquid was deposited in 50 ml tubes and allowed to settle. The sediment at the bottom of the tube was transferred to a Petri dish

containing HTF (Human tubal fluid) with HEPES buffer (*In Vitro* Care®, USA) and albumin. A stereomicroscope was used to search for and select grade 1 cumulus-oocyte complexes (COC) (i.e., compact, dense cumuli with more than three layers and uniform cytoplasm).

The selected COC (average= 140) were placed in a 35 x 10 mm Petri dish with 3 mL maturation medium (BO-IVM). The maturation process was run for 24 h in an incubator (Heraeus™, Germany) under a humidified atmosphere containing 6 % CO_2, at 38.8 °C. Following maturation, those COC exhibiting cumulus expansion were transferred in groups of five into 50-µL drops of mineral oil-coated BO-IVF medium. *In vitro* fertilization (IVF) was performed in BO-IVFTM medium (IVF Bioscience®), following manufacturer instructions. The semen used in the IVF was frozen Red Brangus semen in 0.5-mL straws. Each straw was thawed at 37 °C for 45 sec; the sperm were filtered using Percoll gradients (45%/90% [v/v]) by centrifuging at 328 xg for 3 min. The resulting pellet was mixed with 0.5 mL sperm preparation medium (BO-SemenPrep) and centrifuged at 328 xg for 1 min. The supernatant was discarded and the final pellet mixed with 0.9 ml BO-SemenPrep medium. Sperm concentration was quantified by mixing 25 µL sperm with BO-SemenPrep in 25 µL cold distilled water. From this mixture, 10 µL were placed in a Makler™ counting chamber; the resulting final concentration was 2 x 10^6 sperm/mL.

The oocytes were fertilized with 10 µL (20,000 sperm per 25 ul BO-IVF medium) and incubated for 18 h at 38.8 °C, in a 6 % CO_2 atmosphere with 100 % humidity. After fertilization, the presumed zygotes were stripped of cumulus cells with a 140 µm diameter stripper (MidAtlantic), and washed in BO-IVCTM culture medium (IVF Bioscience). Five presumed zygotes were introduced into 25 µL drops of BO-IVC medium and placed in an incubator (Cook Minc™, USA) at 38.8 °C in an atmosphere containing 5 % CO_2, 6 % O_2 and 89 % N_2[9].

The embryos were evaluated on d 7 and 8, and grade 1 blastocysts selected[10] for bisection. Only the embryos from treatment 2 (n= 30) were used in bisection. Each embryo was placed in a Petri dish with manipulation medium (protein-free PBS). Bisection was done with a microblade adapted to a micromanipulator (M-152 Narishige, USA) placed on an inverted bottom microscope (Olympus CK2, Japan). In bisection, the cut is intended to divide the inner cell mass and the trophoblast into equal parts. The cut was made by slowly lowering the blade in a single movement. PBS supplemented with polyvinyl alcohol (PVA; Vigro, USA) was added to the protein-free medium to allow separation of the hemi-embryos from the Petri dish[11].

Embryo and hemi-embryo vitrification and storage was done with a Cryotop® (Kitazato, Japan)[12]. Two solutions were used: an equilibrium solution (ES), composed of phosphate buffer solution (PBS) with 20 % synthetic serum substitute (SSS), 7.5 % ethylene glycol (EG) and 7.5 % dimethyl sulfoxide (DMSO); and a vitrification solution (VS), composed of PBS with 20 % SSS, 15 % EG, 15% DMSO and 0.5 M sucrose. One drop (20 µL) ES and four drops (20 µL each) VS were placed in a Petri dish at room temperature. Each whole embryo or hemi-embryo was inserted into a single drop of ES and left there for 10-15 min. The embryo or hemi-embryo was transferred to the VS drops, and left for 5, 5, 10 and 10 sec, in succession. A pipette was used to aspirate the embryo or hemi-embryo and place it in the Cryotop® device (Kitazato, Japan). They were immersed in liquid nitrogen and stored in a cryogenic thermos until use[12].

The recipient animals were 60 *Bos taurus*/*Bos indicus* cross cows (mix proportions varied). They were aged 4 to 7 yr, and had a body condition score of 2.5 to 3 (on a scale of 1 to 5). All were clinically healthy, with no history of dystocia, metritis or abortions, no abnormalities in the reproductive tract, and a corpus luteum and/or follicles larger than 10 mm in one of the ovaries on the day of selection (evaluated by ultrasound; Kaixin® model XK5000). The cows were vaccinated one month prior to beginning the synchronization protocol (Cattle Master® 4+L5, Zoetis, Mexico), and dewormed internally (Valbazen 10%, Zoetis, Mexico) and externally (Effipro® Bovis, Virbac, Mexico). During the experimental period, they grazed *Brachicaria brizantha* supplemented with free access to mineral salt. Ovulation synchronization was done by placing an intravaginal progesterone-releasing device (CIDR-B®, 1.9 g P_4, Zoetis, Mexico), and intramuscular injection of 2 mg estradiol benzoate (Bioestrogen® Biogenesis Bagó, Argentina) on protocol day zero. After the CIDR-B® was removed on d 8, an intramuscular injection was applied containing 300 IU equine chorionic gonadotropin (eCG) (Novormon®, Zoetis, Mexico), 25 mg dinoprost tromethamine (Lutalyse®, Zoetis, Mexico) and 1 mg estradiol cypionate (E.C.P.®, Zoetis, Mexico)[13].

Embryo and hemi-embryo transfers were done nine days after intravaginal device removal. The embryos and hemi-embryos were thawed using three solutions: thawing solution (TS), containing PBS with 20 % SSS and 1 M sucrose; dilution solution (DS), containing PBS with 20 % SSS and 0.5 M sucrose; and washing solution (WS), containing PBS with 20 % SSS. To begin, a drop (500 µL) of TS at 37 °C was placed in a Petri dish. Three drops (20 µL each) DS and two drops (20 µL each) WS at room temperature were placed in a second dish. The Cryotop® was uncovered in a cooler containing liquid nitrogen and quickly immersed in the TS drop for 1 min. Using a Pasteur pipette, the embryos and hemi-embryos were placed for 2 min in each DS drop, and 3 min in each WS drop[12]. The embryos and hemi-embryos were placed in PBS and aspirated into 0.25 cc straws; only one whole embryo was placed in a

straw while two hemi-embryos were placed in a straw). A total of 30 vitrified whole embryos and 30 vitrified bisectioned embryos (hemi-embryos) were prepared. These were assigned randomly to the 60 recipient cows; in other words, each cow received either one complete embryo or two hemi-embryos. The transfers were done non-surgically - transcervically in the cows that, upon palpation and rectal ultrasound, exhibited a corpus luteum at least 20 mm in diameter. Five minutes before the transfer, 5 mL 2% anesthesia (100 mg, Lidocaine®, Intervet, Mexico) were applied epidurally to each cow to minimize rectal contractions and facilitate handling of the reproductive system during the transfer[14].

The transcervical transfer was done using a 0.25 ml straw previously loaded with one complete embryo or two hemi-embryos. This was introduced into a metal applicator, which was covered with a sterile sheath and then a plastic sheath. The applicator was introduced into the vagina until reaching the external omentum of the cervix. The cervical canal was traversed by rectal manipulation, guiding the applicator towards the ipsilateral horn of the corpus luteum, where the embryo or hemi-embryos were deposited. Pregnancy diagnosis was done by transrectal ultrasound at 30- and 60-d post transfer, to identify the presence of the amniotic vesicle (30 d) and embryo heartbeat (60 d).

Sample size was calculated by a power analysis run with PROC POWER in the SAS package[15] to calculate treatment frequency. The modeled proportions per treatment were 0.10 and 0.40, considering a test power of 0.80, and a significance level of 0.5. Because the tested treatment was expected to increase pregnancy rate, a one-tailed test with the option sides=1 was used. The estimated number of observations per treatment was 30 animals per group. A completely random design was used with two treatments (without and with bisection) with 30 replicates (recipient cows) each. To identify the statistical relationship between pregnancy rate at 30 and 60 d, a Fisher's exact test for small sample contingency tables was run, using the Fisher option PROC FREQ in SAS[15] and the Weight option to specify observation frequency.

Thirty (30) days post-transfer, no differences (P>0.05) were observed between the whole embryos and hemi-embryos (Table 1). Previous studies have reported a 30 % pregnancy rate with transfer of a single hemi-embryo[14], lower than the 36 % rate observed here with transfer of two hemi-embryos to the same cow. This higher rate may be due to transfer of both hemi-embryos since the number of trophectoderm cells is not reduced by half, as occurs with transfer of a single hemi-embryo. The latter may cause a decline in the amount of interferon tau (produced in trophectoderm cells), a protein associated with maintenance of the corpus luteum and early pregnancy.

Table 1: Pregnancy rate for embryos and hemi-embryos at day 30 post-transfer.

Treatment	N	Pregnancy day 30 (%)	*P* ≤ F	CI95
Embryo	30	20.0	0.0838	0.13 - 0.96
Hemi-embryo	30	36.6		

N = number of observations; *P*= probability value less than F; CI95= 95% confidence interval.

The vitrification technique used here maintained *in vitro*-produced hemi-embryo viability; the same vitrification technique has been used with *in vitro*-produced whole embryos, which were sectioned after thawing. The 33 % pregnancy rate at 60 d observed in the present results is lower than the 46 % at 65 d reported elsewhere(16) (Table 2). This discrepancy could be due to post-thaw sectioning of the embryos in the latter study. Furthermore, the hemi-embryos in this previous study were cultured for 2 h after sectioning, which may have aided pre-transfer re-expansion, improving their viability and the probability of resulting in a pregnancy. In the present study, the embryos were sectioned before vitrification, which could have led to greater exposure to cryoprotectants because, as hemi-embryos, they are not completely covered by a zona pellucida. The decision to section the embryos prior to vitrification was made because this eliminates the need to transport an inverted microscope into the field to finish the post-thaw procedure.

Table 2: Pregnancy rate for embryos and hemi-embryos at day 60 post-transfer

Treatment	N	Pregnancy day 60 (%)	*P* ≤ F	CI95
Embryo	30	16.7	0.0838	0.12 - 0.96
Hemi-embryo	30	33.3		

N = number of observations; *P*= probability value less than F; CI95= 95% confidence interval.

Pregnancy losses between d 30 and 60 post-transfer did not differ (P>0.05) in any of the treatments; in other words, bisection had no effect on pregnancy rate (Table 3). Early pregnancy loss has a major impact on overall transfer success, ranging from 3.1 to 14 %(17,18) with transfer of *in vitro*-produced embryos, and 8.9 to 41 % in cryopreserved ones(17,19,20). The d 30 to 60 pregnancy loss rates in the present results are within previously reported values; indeed, the control group (vitrified whole embryos) had a lower rate than reported elsewhere(17,20). This was probably due to multiple factors, such as the vitrification protocol, culture media, recipients and handling; there is no one factor that determines pregnancy loss rate. The variation in pregnancy loss rates between different studies has been attributed to variability in gene expression, since genes can alter lipid metabolism at the mitochondrial

level, thus increasing cytoplasm lipid droplet content and lowering viability[14,21]. Even *in vitro*-produced embryos considered of excellent quality can experience variations in gene expression that result in failures in embryo development and fetal membranes, causing pregnancy losses[22]. This is one of the principal factors affecting consistent pregnancy rates.

Table 3: Pregnancy loss rate for vitrified embryos and hemi-embryos between days 30 and 60 post-transfer

Treatment	Day post-transfer	N	Pregnancy (%)	$P \leq$ F	IC95
Embryo	30	30	20.0	0.25	0.50 - 0.75
	60	30	16.7		
Hemi-embryo	30	30	36.6	0.21	0.50 - 0.71
	60	30	33.3		

N = number of observations; *P*= probability value less than F; CI95= 95% confidence interval.

In conclusion, the bisection technique used here prior to vitrification with *in vitro*-produced bovine embryos is feasible, but does not improve pregnancy rates compared to vitrified whole embryos. This bisection technique had no effect on pregnancy loss rates compared to the control treatment. The pregnancy rates with hemi-embryos observed here were comparable to those reported for transfer of whole *in vitro* embryos. This highlights that, even after laboratory microsurgery and cryopreservation by vitrification, they can still develop properly without a complete zona pellucida. This has the added benefit of eliminating the need to transport bisection materials and equipment into the field.

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