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Intrafollicular transfer of fresh and vitrified immature bovine oocytes

José Felipe W. Sprícigo [a], Severino Bernardino Sena Netto [a], Carolle Vieira Muterlle [a], Sarah de Andrade Dias Rodrigues [a], Ligiane Oliveira Leme [a], Ana Luiza Guimarães [a], Felippe Manoel Costa Caixeta [a], Maurício Machain Franco [b], Ivo Pivato [a], Margot Alves Nunes Dode [a,b,*]

[a] School of Agriculture and Veterinary Medicine, University of Brasilia, Brasília-DF, Brazil
[b] Embrapa Genetic Resources and Biotechnology, Laboratory of Animal Reproduction, Brasília-DF, Brazil

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ABSTRACT

Embryo production by intrafollicular oocyte transfer (IFOT) represents an alternative for production of a large number of embryos without requiring any hormones and only basic laboratory handling. We aimed to (1) evaluate the efficiency of IFOT using immature oocytes (IFIOT) and (2) compare embryo development after IFIOT using fresh or vitrified immature oocytes. First, six IFIOTs were performed using immature oocytes obtained by ovum pickup. After insemination and uterine flush for embryo recovery, 21.3% of total transferred structures were recovered excluding the recipient's own oocyte or embryo, and of those, 26% (5.5% of transferred cumulus–oocyte complexes [COCs]) were morula or blastocyst. In the second study, we compared fresh and vitrified-warmed immature COCs. Four groups were used: (1) fresh immature COCs (Fresh-Vitro); (2) vitrified immature COCs (Vit-Vitro), with both groups 1 and 2 being matured, fertilized, and cultured *in vitro*; (3) fresh immature COCs submitted to IFIOT (Fresh-IFIOT); and (4) vitrified immature COCs submitted to IFIOT (Vit-IFIOT). Cumulus–oocyte complexes ($n = 25$) from Fresh-IFIOT or Vit-IFIOT groups were injected into dominant follicles (>10 mm) of synchronized heifers. After excluding one structure or blastocyst, the recovery rates per transferred oocyte were higher ($P < 0.05$) for Fresh-IFIOT (47.6%) than for Vit-IFIOT (12.0%). Blastocyst yield per initial oocyte was higher ($P < 0.05$) for Fresh-Vitro (42.1%) than for Fresh-IFIOT (12.9%). Vit-Vitro presented higher ($P < 0.05$) embryo development (6.3%), compared to Vit-IFIOT, which did not result in any extra embryo. Although IFOT did not improve developmental competence of vitrified oocytes, we achieved viable blastocysts and pregnancies produced after IFIOT of fresh bovine immature oocytes. Further work on this technique is warranted as an option both for research studies and for clinical bovine embryo production in the absence of laboratory facilities for IVF.

1. Introduction

The ability to preserve female gametes is an integral part of assisted reproductive techniques as it has a great impact on animal conservation programs, animal breeding programs, and human fertility preservation. [1,2].

To date, the ability of cryopreserved oocytes to achieve later embryonic development is unsatisfactory in most domestic animals. Among the factors responsible for the severe damage caused during cryopreservation [3–10], the high cytoplasm lipid content and the membrane

* Corresponding author. Tel.: +55 61 3448 4659; fax: +55 61 33403658.
E-mail address: margot.dode@embrapa.br (M.A.N. Dode).

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phospholipid composition [11] are often described as responsible for these injuries.

In the last decade, many efforts have been made to increase the vitrification efficiency for farm animal oocytes [1,8], including the use of stabilizing agents for the cytoskeleton [12], antioxidants [13], lipolytic agents [14,15] and plasma membrane modifiers [4,16]. However, no significant improvements have been obtained.

Although no difference in oocyte diameter is evidenced between cattle and humans [11], Cobo et al. [17] and Chang et al. [18] reported that human embryonic production from vitrified oocytes is similar to that obtained from fresh oocytes. These observations indicate that the cell size problems can be overcome and may not be the biggest problem to oocyte cryopreservation. Therefore, we hypothesized that the method of maturation could be an important factor in the poor survival of *in vitro*–matured bovine oocytes subjected to vitrification. In fact, it has been shown in cattle that IVM leads to different lipid and mitochondrial behavior patterns in matured oocytes which can, in turn, alter lipid metabolism affecting cryoresistance [19] However, we found no improvement after vitrification of bovine *in vivo*–matured oocytes (3.6% blastocysts after warming and IVF) in comparison with vitrification of *in vitro*–matured oocytes (2.8%–4.3%) [20].

The *in vitro* system is well known to induce a number of biochemical and morphological changes, such as higher lipid accumulation [21], changes in O_2 consumption [22], and changes in gene expression [23] In addition to the environmental conditions themselves, several specific molecules produced in the reproductive tract of the cow have been shown to be essential for the embryo. Therefore, the development of one entire *in vivo* system, which is the gold standard for embryo production, could be favorable for the vitrified oocytes, allowing them to develop at a higher rate than the *in vitro* environment.

One option to provide *in vivo* conditions for the vitrified oocytes would be to place then back into the female tract by intrafollicular oocyte transfer (IFOT), which was first proposed in baboons and cattle [24] and was then used successfully in mares [25]. This technique would allow the injection of immature oocytes to preovulatory follicles; thus, those oocytes could be matured, fertilized, and cultured in the female tract until the blastocyst stage, when embryos can be recovered by uterine flushing. Hinrichs and DiGiorgio [25] were the first to perform oocyte injection in mares; they injected the oocytes into the follicle with a needle that passed through a cannula placed on the abdominal wall, to puncture the follicle. Subsequently, a transvaginal ultrasound guide was used to localize and guide the needle to perform the injection in mares [26], cattle [27], and even in humans [28]. The first pregnancy and live healthy birth was only recently reported after injection of matured oocytes into the preovulatory follicles of recipients [29]. To the best of our knowledge, there has been no report of pregnancies after transfer of IFOT-derived embryos, when intrafollicular immature oocyte transfer (IFIOT) was used. Also, there is no report of using vitrified and warmed immature oocyte for IFIOT. Our hypothesis was that the entire *in vivo* system using IFIOT would positively affect the oocyte after vitrification and warming. First, a preexperiment was performed to evaluate the feasibility and quality of IFIOT-produced embryos. After this, we compared the developmental ability and embryo outcomes of fresh and vitrified/warmed oocytes submitted to *in vivo* (IFIOT) or *in vitro* system.

2. Materials and methods

Unless otherwise specified, all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cryotop devices were acquired from Dibimed-Biomedical Supply (S.L. Valencia, Spain).

2.1. Ethics

All the experiments, including the preexperiment, were conducted according to Brazilian laws for animal ethics and health research and were approved by the Institutional Animal Care and Use Committee (Embrapa) protocol (process number 005/2015).

2.2. Experimental design

First, a preexperiment was performed to verify the viability of using immature oocytes for intrafollicular transfer. We performed ovum pickup once a week for 3 weeks on five Gir cows (*Bos taurus indicus*). The recovered cumulus–oocyte complexes (COCs) with intact cytoplasm and at least three cumulus cell layers were then used for IFIOT (n = 10–22 per transfer, total of six transfers), in which Nellore (*Bos taurus indicus*) heifers were used as COCs recipients. Note that only recipients that had one dominant follicle at the moment of injections were used. Frozen-thawed semen from a single Gir bull was used for artificial insemination after IFIOT. Subsequently, we recovered bovine morulae and blastocysts by uterine flushing 8 days after IFIOT, corresponding to the seventh day of embryo development (D7). Blastocysts graded as I or II according to International Embryo Technology Society (IETS) were immediately transferred to previously synchronized Nellore heifers, and pregnancy was assessed by ultrasound of the uterus 60 and 90 days after embryo transfer.

Second, COCs were recovered from slaughterhouse ovaries and were selected and distributed into four treatment groups: (1) fresh immature oocytes (Fresh-Vitro); (2) vitrified/warmed immature oocytes (Vit-Vitro), with both group 1 and 2 subjected to IVM, IVF, and IVC; (3) fresh immature oocytes submitted to IFIOT (Fresh-IFIOT); and (4) vitrified/warmed immature oocytes submitted to IFIOT (Vit-IFIOT). A total of 40 Nellore heifers (*Bos indicus*) that were approximately 40-month old and with similar body conditions were used as oocyte recipients. In each replicate, five heifers were previously synchronized and were artificially inseminated with frozen-thawed semen from the same Nellore bull used for the *in vitro* embryo production. At D7 of development (8 days after IFIOT), embryos produced following IFIOT were recovered by uterine flushing and were morphologically evaluated and compared with their *in vitro*–produced counterpart. Expanded blastocysts from all groups were then stained, for assessment of total