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Developmental kinetics of *in vitro*–produced bovine embryos: An aid for making decisions

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ABSTRACT

Embryo developmental kinetics and embryo survival after cryopreservation have been correlated with embryo quality and viability. The main objectives of this work were to analyze developmental ability and quality of *in vitro*–produced bovine embryos in relation to their kinetics and to establish a criterion of quality to predict further viability. Embryos were classified and grouped by their specific stage of development (2, 3–4, or ≥ 5 cells) at 44 hours post insemination (hpi) and cultured separately up to Day 8. On Days 7 and 8, good quality expanded blastocysts were vitrified or frozen. Cryopreserved surviving hatched embryos were stained for cell counts. Embryos at a more advanced stage (3–4 cells, and ≥5 cells) developed to morulae ($P < 0.001$) and blastocysts ($P < 0.01$) at higher rates than those embryos that had cleaved once by 44 hpi. Vitrification improved the hatching rates of blastocysts at 48 hours ($P < 0.001$) when compared with slow-rate freezing within each group of embryos (3–4 cells and ≥5 cells). After vitrification/warming, blastocysts coming from 3- to 4-cell embryos had higher hatching rates at 48 hours than those that came from ≥5-cell embryos. With regard to differential cell counts, no effect of the initial developmental stage was observed after warming/thawing. However, trophectoderm and total cells were higher in vitrified/warmed than in the frozen/thawed embryos ($P < 0.001$). These data show that selecting IVF embryos at 44 hpi, after the evaluation of their *in vitro* embryo development, could be used as noninvasive markers of embryo developmental competence and may help to select IVF embryos that would be more suitable for cryopreservation.

1. Introduction

In recent decades, the process of embryo selection after *in vitro* bovine embryo production (IVP) has been a matter of study and controversy, mainly because embryo selection after IVC remains a subjective process in most mammalian species [1]. The pursuit of objective method(s) to assess embryo quality and viability, and to predict accurately pregnancies leading to healthy offspring from those embryos, is still an ongoing process [2,3]. The search for practical methods that could provide researchers and practitioners with objective data to select the best embryo to transfer is the goal of many studies. There is a general consensus in the field that embryo morphology, development stage, and/or hatching ability, are not definitive criteria for the estimation of bovine embryo quality, either before transfer or as a judge of the *in vitro* embryo production system [4]. From a practical point of view, noninvasive measurements of embryo quality and viability are preferred over invasive methods such as total/differential cell counts [5,6], assessment of apoptotic cells [7,8], and detection of chromosomal abnormalities [9]. The reason is that the former procedures do not allow the subsequent use of analyzed embryos. A potentially useful method to assess chromosomal abnormalities and gene expression is the biopsy of the embryo because it does not prevent the further use of the embryos. However, embryo viability may be compromised after this procedure [10]. Therefore, noninvasive markers of embryo developmental

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competence are needed [1]. Different approaches of noninvasive methods to assess embryo quality have been proposed such as metabolic tests [11–15], evaluation of embryo survival after cryopreservation [16–18], assessment of individual embryo culture medium using Fourier transform infrared metabolomics [19,20], and embryo developmental kinetics. Although it is well known that culture conditions influence the kinetics of embryo development [21–24], other factors may also be involved. The timing of certain events during the earliest stage of development may be linked to subsequent embryonic viability [25]. In this regard, the timing of the early cleavage events is a critical parameter that may be more informative than the conventional approach of simply recording the overall cleavage at one timepoint after insemination or the percentage of embryos developing to the blastocyst stage [26]. Moreover, early cleaving bovine embryos selected according to the time of completion of the first division have a greater development potential than late cleaving embryos [27–30]. In addition, Somfai et al. [31] concluded that the lengths of the first and second embryonic cycles and the cleavage pattern during the first cell division are potent predictors of developmental competence and should be viewed simultaneously for the selection of good quality embryos for embryo transfer.

Cryopreservation survival rates are among the most important parameters of quality [17] and are useful in assessing embryo viability. There is evidence that indicates that the fastest developing embryos are more tolerant to cryopreservation procedures than the embryos that develop more slowly [30] and are more likely to survive cryopreservation with the establishment of pregnancies after embryo transfer [16]. In a recent article, we reported that vitrification was a better alternative than slow-rate freezing for cryopreserving IVP bovine blastocysts under our laboratory conditions [32]. However, we did not consider the kinetics of embryo development. Regular evaluation of embryo development is time-consuming, and under laboratory/field conditions, a more practical approach is needed. In this article, we selected and classified the embryos at a specific timepoint after fertilization depending on their developmental stage, and the subsequent development and survival rates after cryopreservation were assessed.

The objectives of this research were to analyze developmental ability and the quality of *in vitro*–produced bovine embryos in relation to their kinetics, and to establish a criterion of quality and viability to help us select the best embryos for their subsequent use.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma (Madrid, Spain), unless otherwise indicated.

2.2. In vitro embryo production

Cumulus–oocyte complexes (COCs) from slaughterhouse ovaries were aspirated from 3 to 8 mm visible follicles and rinsed three times in a holding medium (HM) consisting of TCM199 (Invitrogen, Barcelona, Spain), 25-mM HEPES, and BSA 0.4 g L^{-1}. Oocytes enclosed in a compact cumulus with an evenly granulated cytoplasm were selected and washed twice in maturation medium, which consisted of bicarbonate-buffered TCM199, porcine FSH-LH (1:5 μg mL^{-1}, Stimufol; ULg FMV, Liège, Belgium), 17β-estradiol (1 μg mL^{-1}), and 10% fetal calf serum (FCS). Maturation was performed by culturing approximately 50 COCs in 500 μL of maturation medium in four-well dishes at 39 °C in air with 5% CO_2 at high humidity for 22 to 24 hours.

For IVF, sperm separation was performed following a swim-up procedure [33]. Briefly, semen from one frozen straw (sperm from two bulls was used for the entire study) was thawed in a water bath and added to a polystyrene tube containing 1 mL of pre-equilibrated Sperm-TALP. After 1 hour of incubation, the upper layer of supernatant containing motile sperm was removed. The spermatozoa were centrifuged for 7 minutes at 200 × *g*, and the supernatant was aspirated to leave a pellet containing sperm cells, the concentration of which was determined using a hemocytometer. After washing the COCs in HM, they were rinsed twice in fertilization medium (Fert-TALP) and placed in four-well culture dishes containing the same medium plus 10 μg mL^{-1} heparin (Calbiochem, La Jolla, CA, USA). Spermatozoa were added at a concentration of 2×10^6 cells mL^{-1} in 500 μL of medium per well, each containing a maximum of 100 COCs (Day 0). *In vitro* fertilization was accomplished by incubating the oocytes and sperm cells together for 18 to 20 hours at 39 °C in air with 5% CO_2 at high humidity.

For IVC, cumulus cells were detached using a vortex, and presumptive zygotes were cultured in synthetic oviduct fluid containing amino acids, citrate, and myoinositol—as described by Holm et al. [34]—plus 6 g L^{-1} BSA (mSOF). Droplets (1–2 μL/embryo) were covered by a mineral oil layer, and the embryos were cultured in groups of 35 to 45 at 39 °C in a 5% CO_2/5% O_2 atmosphere at high humidity. The culture medium was renewed on Days 3 and 6 by transferring the embryos to fresh droplets. Embryo development was recorded on Days 2, 6, 7, and 8.

2.3. Embryo vitrification and freezing

Well expanded Day 7 and Day 8 blastocysts were subjected to either vitrification or slow-rate freezing. Vitrification was performed in fibreplugs (CryoLogic vitrification method) as previously described [35]. Briefly, embryos were first washed in a HM consisting of TCM 199-HEPES + 20% FCS. All procedures were performed in a warm room (30 °C) on a heated surface (41 °C). Groups of 1 to 5 blastocysts were exposed to HM with 7.5% ethylene glycol (EG) + 7.5% dimethyl sulfoxide (vitrification solution-1) for 3 minutes and then moved into a microdrop containing HM with 16.5% EG + 16.5% dimethyl sulfoxide + 0.5-M sucrose (vitrification solution-2; VS2). Blastocysts were then loaded into a micropipette in 0.3 to 3.0 μL of VS2 (1 embryo/0.3 μL of VS2) and placed in a microdrop in the hook of the fibreplug. They were then vitrified by bringing the hook into contact with a supercooled block placed in liquid nitrogen. The time spent by the embryos in VS2 (including loading) was 20 to