Cryobiology 73 (2016) 203–208

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Correlation between the cryosurvival, cell number and diameter in bovine in vitro produced embryos

Alper Kocyigit [a, *], Mesut Cevik [b]

[a] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Cumhuriyet University, Sivas, Turkey
[b] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey

ARTICLE INFO

Article history:
Received 7 June 2016
Received in revised form
17 July 2016
Accepted 19 July 2016
Available online 20 July 2016

Keywords:
Bovine blastocyst
Diameter
Vitrification
Cell allocation
Cryosurvival

ABSTRACT

The selection of quality embryos is a prerequisite of cryopreservation process. Present study was conducted to examine the correlation between the diameter and cryotolerance, on the cell number of the cryopreserved embryos. The blastocyst stage embryos were collected at culture days 7–9, evaluated morphologically under a microscope and divided according to the diameter into three groups: Group 1; (larger than 150 μm), Group 2; (diameter of 100–150 μm), Group 3; (smaller than 100 μm). Blastocysts were vitrified-thawed using the classical vitrification method and then cultured in SOF medium drops at 24 h. Blastocysts were considered viable if they re-expanded or hatched from the zona pellucidae. Finally re-expanded blastocysts from the Group 1 and Group 2 to determine the differential count of cells in the ICM and TE. The re-expansion ability of blastocysts 100–150 μm in diameter (69.56%) was significantly higher than other groups (52.17 and 47.36%). The value of the correlation coefficient between the re-expansion rate and cell number of blastocysts in the group 2 ($r = 0.784$) tended to be higher than that in the group 1 ($r = 0.512$) and group 3 ($r = 0.491$) ($p < 0.05$). For ICM/total cell ratio yield group 2 embryos showed higher rate (0.28), compared to the other groups (0.19 and 0.16). In conclusion, the present study demonstrates that the correlation between diameter of embryos and their cryosurvival based on re-expansion ability and cell allocation.

1. Introduction

Improvements in cryobiology provide new opportunities for reproductive biotechnology. Cryopreservation of preimplantation embryos is an important tool for maintaining animal genetic resources by increasing the usage of reproductive potential of genetically valuable animals. However, cryopreservation procedures may negatively affect survivability of gametes and embryos as a consequence of damages to the cell and even cell death [1]. One possible reason for reduced embryo viability following freezing and thawing could be the disruption of the cytoskeleton, as a result of intracellular ice formation [17]. The quality of in vitro produced (IVP) bovine embryos have been widely investigated by evaluating in vivo embryonic development by the transfer to bovine recipients. However, it is well known that pregnancy rate of bovine IVP embryos is lower than that of in vivo derived embryos [9].

The ability to cryopreserved embryos, without critical loss of viability, has a profound effect on the success of assisted reproductive techniques [5,26]. The viability of bovine IVF embryos has been found to be affected by the embryo age, stage of embryonic development and embryo quality [7]. Therefore, selecting embryos by their quality is a prerequisite of cryopreservation process and embryonic morphological appearance is the most common indicator to assess embryo quality. The diameter of the blastocysts, one of the morphological criteria, is positively dependent on the blastomeres size, which may contribute to the efficiency of embryo development. In addition, the cell allocation of the embryos is a indicator of the viability of pre-implantation embryos [24]. It was observed that embryos developing quickly to the blastocyst stage had a higher total cell number than embryos developed more slowly. Therefore, the cell number of bovine IVF blastocysts varied depending on the morphological grade and that the later developing blastocysts were of poor quality as judged by the cell number [13]. Moreover, Iwasaki et al. [12] also demonstrated that the cell numbers of the embryos were different at each stage of embryonic development. Therefore, if there is a correlation between the cell number and diameter of the embryos, thus; the diameter of the

* Corresponding author.
E-mail address: akocyigit@cumhuriyet.edu.tr (A. Kocyigit).

http://dx.doi.org/10.1016/j.cryobiol.2016.07.010
0011-2240/

embryos may be used as indicator for embryo viability after cryopreservation. However, there is very little information regarding the correlation between the cell number and diameter in bovine IVF embryos [19].

Cryotolerance is another useful indicator of blastocyst quality [20]. IVP embryos are more sensitive to cryoinjury than in vivo-derived embryos, such as shown by less compaction, lower number of tight-junctions and especially lower cell numbers compared to that of in-vivo derived embryos [30]. Cell tight-junction formation is a prerequisite for blastocoel formation, blastocyst expansion and differentiation into two distinct cell populations: the trophectoderm (TE) and the inner cell mass (ICM). It is well known that the ratio and allocation of these cells is considered to be a potential indicator of embryo quality and crucial for implantation [28]. The quality of the bovine IVP blastocyst has been associated with their sensitivity to cryopreservation [16] and to the amount and proper allocation of embryonic cells [28]. Blastocyst cell numbers are usually reduced in IVP embryos as compared to in vivo-derived embryos [12,18]. However, there is no useful information regarding the correlation between embryo diameter and cryotolerance based on cell allocation in vitrified bovine embryos. Therefore, in the present study was evaluated the correlation between embryo diameter and cryotolerance to vitrification of bovine IVP blastocyst and whether the allocation of ICM and TE cells influence cryosurvival.

2. Materials and methods

All chemicals used in this study were purchased from Sigma-Aldrich (Istanbul, TURKEY), except where otherwise indicated.

2.1. Collection and in vitro maturation of oocytes

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory at approximately 34 ± 2. °C in physiological saline solution supplemented with gentamycin sulphate (0.1 μl/ml). Cumulus oocyte complexes (COCs) were recovered from follicles 2–8 mm in diameter by aspiration. The COCs were collected in 3–4 ml Hepes-buffered Medium-199 containing Earle's salts and supplemented with 1% v/v antibiotic-antimycotic solution. Before in vitro maturation, the COCs were assessed morphologically and only oocytes with compact, non-atretic cumulus and evenly granulated cytoplasm were selected for in vitro oocyte maturation. Maturation medium was sodium bicarbonate-buffered Medium-199 with sodium pyruvate (5.5 μg/ml), and supplemented with antibiotic-antimycotic solution (1% v/v), heat-inactivated fetal calf serum (FCS, 10% v/v). COC's were matured for 22 h in a humidified atmosphere of 5% CO_2 in air at 38.5 °C.

2.2. Spermatozoa preparation and in vitro fertilization

After a 22 h maturation period, COC's were transferred into 44 μl drops of fertilization medium consisted of glucose-free modified Tyrode's albumin lactate pyruvate (TALP) supplemented with bicarbonate (25 mM), Na-lactate (22 mM), Na-pyruvate (1 mM), fatty acid-free bovine serum albumin (BSA) (6 mg/ml), and heparin-sodium salt (184 units/mg heparin, 10 mg/ml) and antibiotic-antimycotic solution (0.5 μl/ml) (pH 7.4 and 280–300 mOsm/kg). Frozen-thawed bull semen was used for the fertilization of COC's. Percoll density gradient system was used for the separation of the motile fraction of the frozen-thawed semen [25]. Sperm were then diluted to 50 × 10^6 spermatozoon/ml in TL-HEPES, including the 2 × 10^6 motile spermatozoa/ml as final concentration. The fertilization procedure was completed by adding 2 μl of diluted sperm, 2 μl heparin (5 μg/ml) and 2 μl of PHE solution (20 μM penicillamine, 10 μM hypotaurine, and 1 μM epinephrine in final concentration) into the fertilization drops containing oocytes. COC's were fertilized with 2 μl diluted semen per fertilization drops for 22 h in a humidified atmosphere of 5% CO_2 in air at 38.5 °C.

2.3. In vitro culture

Cumulus cells surrounding the oocytes were removed from presumptive zygotes at approximately 22 h post-insemination by vortexing 3 min. The zygotes were transferred in groups of 20–30 per drop and cultured in synthetic oviduct fluid (SOF) medium supplemented with pyruvate (0.4 mM), fatty acid free (BSA-FAF) (8 mg/ml), 100× minimum essential medium (MEM) (20 μl/ml), 50× basal medium eagle (BME) (10 μl/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) on the day of use a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C.

In the present study, IGF-1 (100 ng/ml) was used to supplement the culture medium following our study of previous reports at Day 0 (fertilization day) [15].

Blastocysts were collected at days 7–9 of in-vitro culture and morphologically evaluated by visualizing presence of the blastocele and distinct ICM. Blastocysts were classified according to their diameter into three groups: Group 1: Expanded Blastocyst; where the diameter of the embryo was larger than 150 μm and the thickness of the zona pellucidae could be reduced to approximately 1/2 of the original thickness, Group 2: Normal Blastocyst; where the blastocoel was visible, the ICM was becoming distinct, and the overall embryo diameter was between 100 and 150 μm. Group 3: Early Blastocyst; where the embryo diameter was smaller than 100 μm. The embryo diameter was measured with an ocular micrometer attached to an inverted microscope (Nikon, ECLIPSE, and TS100). The ocular micrometer was calibrated at ×20 objective and ×10 eyepiece. The embryo diameter (including the zona pellucidae) was calculated by the mean of two measurements made perpendicular to each other.

Total blastocysts were separated into two groups: Experiment 1 control group (non-vitrification), and Experiment 2: vitrification group.

2.4. Vitrification and warming

The vitrification procedure is based on the classical straw method with minor modifications [11]. After two initial washes in SOF medium, blastocysts were placed in a 10% glycerol solution for 1 min, then transferred to 20% ethylene glycol (EG) + 10% glycerol solution for 1 min and finally transferred to %25 EG + %25 glycerol for 25 s. Blastocysts were immediately drawn by capillary action into sterile straws (0.25 ml), along with a maximum of 1–2 ml medium. Blastocysts were vitrified using the classical method of directly submerging embryos into liquid nitrogen after brief exposure to a cryoprotectant solution. For warming (one day after cryopreservation), straws were thawed in a water bath at 35 °C for 15 s, and their contents released into 0.5 M sucrose solution. Thawed blastocysts were recovered and equilibrated in 0.5 M and 0.25 M sucrose for 5 min, each and then washed once in SOF medium containing BSA-FAF (8 mg/ml) and cultured in SOF drops for 24 h. Blastocysts were considered that they survived the vitrification (viable) if they re-expanded or hatched from the zona pellucidae after in vitro cultured.

2.5. Differential staining

To determine the differential cell count of TE and ICM, re-expanded blastocysts from Experiment 1 and 2 were differentially stained as described by Soom et al. [28]. Briefly, zona intact