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Technical note

The influence of cumulus cells during *in vitro* fertilization of buffalo (*Bubalus bubalis*) denuded oocytes that have undergone vitrification

Laura Attanasio, Anna De Rosa, Marina De Blasi, Gianluca Neglia*, Luigi Zicarelli, Giuseppe Campanile, Bianca Gasparrini

DISCIZIA, Faculty of Veterinary Medicine, "Federico II" University, Via F. Delpino 1, 80137 Naples, Italy

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Abstract

The aim of this work was to evaluate whether providing a support of cumulus cells during IVF of buffalo denuded oocytes submitted to vitrification-warming enhances their fertilizing ability. *In vitro* matured denuded oocytes were vitrified by Cryotop in 20% EG + 20% of DMSO and 0.5 M sucrose and warmed into decreasing concentrations of sucrose (1.25 M–0.3M). Oocytes that survived vitrification were fertilized: 1) in the absence of a somatic support (DOs); 2) in the presence of bovine cumulus cells in suspension (DOs+susp); 3) on a bovine cumulus monolayer (DOs+monol); and 4) with intact bovine COCs in a 1:1 ratio (DOs+COCs). *In vitro* matured oocytes were fertilized and cultured to the blastocyst stage as a control.

An increased cleavage rate was obtained from DOs+COCs (60.9%) compared to DOs, DOs+susp (43.6 and 38.4, respectively; $P < 0.01$) and DOs+monol (47.5%; $P < 0.05$). Interestingly, cleavage rate of DOs+COCs was similar to that of fresh control oocytes (67.8%). However, development to blastocysts significantly decreased in all vitrification groups compared to the control ($P < 0.01$).

In conclusion the co-culture with intact COCs during IVF completely restores fertilizing capability of buffalo denuded vitrified oocytes, without improving blastocyst development.

Keywords: Buffalo; Oocyte vitrification; Cumulus cells

1. Introduction

Interest in oocyte cryopreservation in buffalo has increased with the improving efficiency of the *in vitro* embryo production (IVEP) technology in this species [1]. Indeed, oocyte cryopreservation is critically important in buffalo species because the major limitation to the diffusion of IVEP technology in the field is the low number of oocytes recovered per ovary [2]. However, buffalo oocytes are highly sensitive to chilling injuries because of their high intracytoplasmic lipid content [3].

In vitro matured buffalo oocytes have been successfully cryopreserved by ultrarapid vitrification methods, such as Solid Surface Vitrification, Cryoloop and Cryotop [3–5]. However, despite the high post-warming survival rates, cleavage and blastocyst yields after IVF are still poor [3,4].

Among others, a critical factor that may affect oocyte vitrification efficiency is the removal of cumulus cells. It has been hypothesized that cumulus cells and glycoproteins slow cryoprotectants penetration that

* Corresponding author. Tel.: 39-081-2536063; fax: 39-081-292981.
E-mail address: neglia@unina.it (G. Neglia).

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may lead to unequal intracellular distribution of the cryoprotectant and inadequate cell protection [6]. It was previously demonstrated that removal of cumulus cells prior to vitrification is advisable for oocyte vitrification in buffalo [3]. On the other hand, when denuded oocytes are vitrified their embryo development is in part affected by the lack of cumulus cells during the process of fertilization, as previously demonstrated for non vitrified oocytes in this species [3,7]. Cumulus cells play an important role during fertilization by attracting, trapping, and selecting spermatozoa [8,9], facilitating sperm capacitation, acrosome reaction and penetration [9,10] and preventing precocious zona pellucida hardening [11]. The latter phenomenon is known to occur after cryopreservation, leading to reduced fertilization rates [12,13].

A partial reduction of the cumulus cell layers before vitrification, that may represent the solution to this problem, is difficult to carry out in buffalo species because of the lower adhesion of cumulus cells to the oocyte [2]. In earlier times different co-culture systems have been used for in vitro culture of buffalo embryos [2]; furthermore, in our experience buffalo embryos have been successfully co-cultured on a monolayer of bovine cumulus cells (unpublished results).

Based on these observations, the aim of this work was to evaluate whether inseminating vitrified-warmed denuded oocytes in co-culture with cumulus cells restores their capability to cleave and to develop into blastocysts. In particular, we analyzed the efficiency of co-culture with bovine cumulus cells 1) in suspension, 2) as a monolayer, and 3) as intact bovine COCs, during IVF of buffalo oocytes freed of cumulus investment prior to vitrification.

2. Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.1. Oocyte collection and IVM

Abattoir-derived cumulus–oocyte complexes (COCs) were recovered by aspiration of 2–8 mm follicles using an 18-G needle under vacuum (40–50 mmHg). COCs with a compact, non-atretic cumulus and a homogeneous cytoplasm were matured (10 COCs per 50 μL drop under mineral oil) in TCM199 buffered with 25 mM sodium bicarbonate and supplemented with 10% fetal calf serum (FCS), 0.2 mM sodium pyruvate, 0.5 μg/mL FSH, 5 μg/mL LH, 1 μg/mL 17β-estradiol, 50 μg/mL kanamycin, 50 μM cysteamine, and 0.3 mM cystine. The IVM was carried out at 38.5 °C in a controlled gas atmosphere of 5% CO_2 in humidified air for 21 h.

2.2. Vitrification and warming

After IVM, the cumulus cells were mechanically removed by gentle pipetting in the Hepes-buffered TCM199 (H199) with 20% FCS and the denuded oocytes (n = 640) were vitrified using the Cryotop tool [14] with the cryoprotectants concentrations, equilibration times and warming procedures previously used for buffalo oocytes [4]. All equilibration and dilution steps were carried out at room temperature. Media for vitrification/warming were made in H199 + 20% FCS. Briefly oocytes were first exposed for 3 min to 10% ethylene glycol (EG) + 10% DMSO and then transferred to the final vitrification solution, consisting of 20% EG + 20% DMSO and 0.5 M sucrose for 20–25 sec. Five oocytes were loaded in < 0.1 μl volume onto the top of the film strip of each Cryotop that was quickly immersed into liquid nitrogen, protected with the cap and stored under liquid nitrogen for 1 week. For warming, the Cryotops were first transferred into a 1.25 M sucrose solution for 1 min, subsequently exposed to decreasing concentrations of sucrose (0.62 M, 0.42 M and 0.31 M) for 30 sec each. Oocytes were then washed in H199 + 10% FCS and allocated into the IVM drops for 1.5 h, after which survival rate was evaluated on the basis of the integrity of the oocyte membrane and the zona pellucida together with the homogeneity of the cytoplasm. The oocytes that survived were then fertilized *in vitro*.

2.3. In vitro fertilization and in vitro culture

Frozen–thawed sperm of a bull previously tested for IVF were treated by swim-up procedure in Hams F-10 medium for 1 h. The sperm pellet obtained after centrifugation of the supernatant was re-suspended to a final concentration of 2×10^6 mL^{-1} in the IVF medium, i.e. Tyrode albumin lactate pyruvate [15] supplemented with 0.2 mM penicillamine, 0.1 mM hypotaurine, and 0.01 mM heparin. Insemination was performed in 50 μl drops of IVF medium under mineral oil (5 oocytes per drop) at 38.5 °C under humidified 5% CO_2 in air for 22 h. After IVF, oocyte survival rate was reassessed (survival at 24 h post-warming) and the oocytes that had survived were allocated into 20 μl drops of SOF medium [16]. Culture was carried out under humidified 5% CO_2, 7% O_2, and 88% N_2 at 38.5 °C. Cleavage and blastocyst rates were assessed on Day 5 and 7, respectively (Day 0 = IVF).