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The use of coconut water solution (*Cocos nucifera*) as a holding medium for immature bovine oocytes for in vitro embryo production

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Abstract

The aim of this study was to evaluate a coconut-water-based holding medium for preserving immature bovine cumulus-oocyte complexes (COCs). The selected COCs were grouped as follows: Control Group – immediately-matured COCs; Groups H6, H9, and H12 - COCs preserved in TCM-199 + Hepes for 6, 9, and 12 h, respectively; and Groups C6, C9, and C12 - COCs preserved in the holding coconut solution for 6, 9, and 12 h, respectively. The total *in vitro* maturation (IVM) time of the experimental groups was 24 h, that is, preservation for 6, 9, and 12 h and IVM for 18, 15, and 12 h, respectively. After *in vitro* fertilization and embryo culture, cleavage, blastocyst, and hatched blastocyst rates were analyzed. A decrease was observed ($P < 0.05$) in blastocyst rates in Groups H9 (33.9 ± 8.7%), H12 (34.2 ± 6.0%), C9 (35.9 ± 8.9%), and C12 (35.6 ± 5.6%) when compared to the Control (50.9 ± 8.7%), H6 (49.7 ± 7.3%), and the C6 Groups (49.4 ± 4.1%). The results indicate that the coconut-water holding solution can be used as a medium to preserve immature bovine COCs for up to 6 h with no effects on embryo production.

Keywords: coconut water, oocyte, *in vitro* embryo production.

Introduction

Keeping cumulus-oocyte complexes (COCs) viable during transportation from farms to laboratories is fundamental for successful commercial *in vitro* embryo production (IVEP) because preservation conditions may affect the developmental capacity of the oocytes (Schwartz *et al*., 1998; Ward *et al*., 2000). Several culture media for the preservation of COCs, such as Ham F-10, Tissue Culture Medium (TCM 199) with and without Hepes buffer, Tyrode's medium with albumin, lactate and Pyruvate buffered with Hepes (TALP-Hepes), and follicular fluid itself (Twagiramungu *et al*., 1998; Leivas *et al*., 2001; Alves *et al*., 2003) have been evaluated for different periods and at different temperatures. Currently, the main preservation medium used in the transport of COCs from farms to laboratories is TCM 199–Hepes (Alves *et al*., 2003; Leivas *et al*., 2004); however, its high cost and mainly its low viability over long transportation periods (Twagiramungu *et al*., 1998; Leivas *et al*., 2004) in suboptimal conditions are limiting factors for commercial use with IVEP. This has led researchers to investigate alternative, inexpensive, and easily obtainable media to ensure greater COC viability.

Coconut water has been extensively tested and used as an extender of semen for several animals such as pigs (Toniolli and Mesquita, 1990), goats and sheep (Nunes, 1997), buffalo (Vale *et al*., 1999), dogs (Cardoso *et al*., 2003; Cardoso *et al*., 2006) and humans (Nunes, 1998). It has also been investigated as a maturation and culture medium for bovine oocytes and embryos (Blume *et al*., 1997a; b). Coconut water has been used in the culture of preantral follicles of goats (Silva *et al*., 1999; 2000; 2004), sheep (Andrade *et al*., 2002), and cattle (Lucci *et al*., 2004), as well as a culture medium for microorganisms (*Mycobacterium tuberculosis*; Vasanthakumari and Jagannath, 1998), which demonstrates the versatility of coconut water as a preservation and culture medium of different cells types. The objective of this work was to evaluate the use of a coconut-water-based solution as a preservation medium for immature bovine COCs for use with *in vitro* production of embryos

Materials and Methods

COC collection and selection

Bovine ovaries supplied by a slaughterhouse were punctured with 18-g 'needles and 10 ml syringes. The follicular fluid was collected and placed in 15-ml tubes for sedimentation. COCs were inspected under laminar flow with a stereomicroscopy magnifying glass, and only COCs with a homogeneous cytoplasm, without vacuoles or dark granules, and with a compact and refringent cumulus oophorus were selected. Cumulus-oocyte complexes were then washed with TCM-199 + Hepes and 10% fetal calf serum (FCS), 0.2 mM pyruvate, 62.6 µg/ml penicillin, 50 µg/ml gentamicin (TCM-199 + Hepes), and randomly assigned to experimental groups.

Experimental groups

Culumus-oocyte complexes were preserved in microcentrifuge tubes at 30°C and protected from light with aluminum foil. Three replicates were conducted with 20 to 25 COCs in 1 ml preservation medium.

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The selected COCs were randomly assigned to the following experimental groups.

Control Group: *in vitro* mature COCs after selection.

Groups H6, H9, and H12: COCs preserved in TCM-199 + Hepes for 6, 9, and 12 h, respectively.

Groups C6, C9, and C12: COCs preserved in coconut water solution made up of 75 ml of filtered coconut water, 25 ml of ultrapure water and 25 mM Hepes buffer (final osmolarity: 290 mOsmol/L and pH: 7.2) added with 10% FCS (v/v), pyruvate and antibiotics for 6, 9, and 12 h, respectively.

In vitro maturation

Cumulus-oocyte complexes were incubated in IVM (TCM 199 supplemented with sodium bicarbonate, 10% FCS (v/v), 5 µg/ml FSH, 50 µg/ml LH, 10 µg/ml insulin, 50 µM β-Mercaptoetanol, pyruvate, and antibiotics) after specific preservation periods. Incubation was carried out in Petri dishes with 100 µl of IVM (10-13 COCs per drop) under sterile mineral oil in a culture incubator with 5% CO_2 in air at 38.5°C and saturated humidity. The total IVM time was 24 h, that is, IVM for 18, 15, and 13 h and preservation for 6, 9, and 12 h, respectively.

In vitro fertilization

Frozen semen from a single European bull (Bos taurus taurus) was used for *in vitro* fertilization. Cryoprotected spermatozoa and seminal plasma were separated by noncontinuous density (Percoll). After 20 min of centrifugation in Percoll (200 xg), the supernatant was discarded and the pellet was centrifuged for 5 min (200 xg) in 2 ml TALP with Hepes buffer (Bavister *et al*., 1983) and without BSA, glucose, pyruvate, and antibiotics to remove residual Percoll.

Sperm concentration was determined with a Neubauer camera set at 2 x 10^6cell/ml. Fertilization was carried out in TALP-FERT supplemented with heparin, penicillamine, hypotaurine, epinephrine, and BSA according to Parrish *et al*. (1988). Oocytes and spermatozoa were co-incubated in conditions similar to those of IVM for 32 h.

In vitro culture

A bovine embryo co-culture system with a monolayer of granulosa cells from the cumulus oophorus was prepared. The IVM drop medium was replaced with 50 µl of Charles Rosenkrans (CR_2) culture medium (Rosekrans and First, 1991) supplemented with 10% FCS (v/v), BSA, and antibiotics. After IVF, the probable zygotes were submitted to successive pipetting to remove the remaining cumulus oophorus cells and then transferred to culture drops (20 zygote per drop) where they stayed for 9 d. After 72 h from the start of the embryo culture, 50 µL of CR_2 medium was added to the culture medium, and on the 5th day (120 h), 50% of the culture medium was renewed.

Three evaluations were made for the quantitative analysis of pre-implantation embryo development (cleavage, blastocyst, and hatching) according to the pattern established by the International Embryo Transfer Society (IETS) Manual (Stringfellow and Seidel, 1999).

Statistical analyses

The results are expressed as mean ± standard deviation and were analyzed using the BioEstat 3.0 software (Ayres *et al*. 2003). Cleavage (%), blastocyst (%), and hatched blastocyst (%) rates were transformed to arcsine and evaluated by analysis of variance (ANOVA - Bonferroni post-test; significance level of $P < 0.05$).

Results

Embryo development was analyzed in triplicate, totaling 535 COCs across all experimental groups. There was no difference between cleavage and hatching rates ($P > 0.05$) among treatments (Table 1). There was a lower ($P < 0.05$) blastocyst rate in H9 (33.9 ± 8.7%), H12 (34.2 ± 6%), C9 (35.9 ± 8.9%), and C12 (35.6 ± 5.6%) when compared to the Control Group (50.9 ± 8.7%) as well as H6 (49.7 ± 7.3%) and C6 (49.4 ± 4.1%).

Table 1. *In vitro* embryo development of oocytes preserved in either coconut water solution or TCM-199 + Hepes for 6, 9 and 12 h.

Group	No. COCs	Preservation + IVM (h)	Cleavage (%)	Blastocyst (%)	Hatching* (%)
Control	116	0 + 24	79.5 ± 3.0[a]	50.9 ± 8.7[a]	70.1 ± 6.0[a]
H6	73	6 + 18	75.9 ± 3.7[a]	49.7 ± 7.3[a]	69.3 ± 5.8[a]
H9	67	9 + 15	74.9 ± 11.5[a]	33.9 ± 8.7[b]	74.2 ± 10.1[a]
H12	71	2 + 12	71.2 ± 9.2[a]	34.2 ± 6.0[b]	50.5 ± 6.3[a]
C6	72	6 + 18	80.2 ± 3.0[a]	49.4 ± 4.1[a]	78.7 ± 10.7[a]
C9	68	9 + 15	78.1 ± 5.6[a]	35.9 ± 8.9[b]	69.4 ± 12.5[a]
C12	68	12 + 12	75.3 ± 4.6[a]	35.6 ± 5.6[b]	71.5 ± 15.9[a]

[a,b]Different superscripts in the same column differ ($P < 0.05$). Control (COCs submitted directly to IVM); H6, H9, and H12 (COCs preserved in TCM-199 + Hepes); C6, C9, and C12 (COCs preserved in coconut water solution).
*Number hatched / total blastocysts.

Discussion

The preservation of immature bovine COCs for up to 6 h in these conditions indicated that both coconut water solution and TCM-199 + Hepes are viable culture media. Similar results were obtained by Twagiramungu *et al*. (1998) with TCM 199 and TCM-199 + Hepes at 38.5°C and Leivas *et al*. (2001) with TCM 199 at 39°C. However, the preservation media were supplemented with hypophyseal hormones in both cases. Alves *et al*. (2003) also obtained higher development rates in similar conditions to those of the current experiment by using bovine follicular fluid as a preservation medium.

The preservation periods 9 and 12 h did not influence either cleavage or hatching rates compared to those of the Control Group. However, a decrease in embryo development up to the blastocyst stage was observed, regardless of the preservation medium used. This was probably due to the conditions used in the current experiment. This seems to be corrected by the use of hypophyseal hormones and appropriate temperatures, as observed by Leivas *et al*. (2004), who obtained more satisfactory production of embryos after the preservation of bovine COCs in TCM 199-Hepes supplemented with FSH at 39°C for up to 12 h.

Resumption of meiosis occurs spontaneously after the removal of COCs from the follicular medium (Bevers *et al*., 1997). The use of substances that inhibit or at least slow down the rupture of the germinative vesicle (GVBD; Lonergan *et al*., 1997) in the preservation medium can be an alternative for transport of COCs for extended periods of time in commercial IVEP programs. According to Takami *et al*. (1999), antioxidants play an important role in blocking the meiosis. However, ascorbic acid, an antioxidant found in coconut water, had only inhibitory effect when injected directly into the oocyte (Guarnaccia *et al*., 1997). Another alternative would be complementing the preservation medium with hormones (FSH, LH, insulin), growth factors (EGF, IGF), and antioxidants in an attempt to ensure appropriate *in vitro* maturation during the preservation period. However, depending on the medium volume of medium utilized, the use of these additives may even increase cost, a limiting factor of commercial IVEP.

The use of less expensive and equally efficient media such as coconut water solution compared to TCM-199 + Hepes may ensure a significant reduction in the cost of the preservation medium. However, further study on the supplementation of coconut water solution, mainly with hormones, is necessary for transport times longer than 6 h.

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