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A comparative analysis of the efficacy of three cryopreservation protocols on the survival of *in vitro*-derived cattle embryos at pronuclear and blastocyst stages

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ABSTRACT

The effectiveness of three cryopreservation protocols (slow freezing, short equilibration vitrification and long equilibration vitrification) on *in vitro*-derived cattle embryos at expanded blastocyst and pronuclear stages was compared. 199 expanded blastocysts of good quality were assigned randomly into four treatment groups [control, non-cryopreserved (fresh, unfrozen); and the three cryopreservation methods]. The re-expansion of the cryopreserved blastocysts after 24 h *in vitro* culture was similar to that of the fresh control group. However, the hatching rate of expanded blastocysts after 48 h culture was significantly less for the slow freezing group (31/47; 66.0%) than for both the short equilibration vitrification (46/51; 90.2%) and long equilibration vitrification groups (42/50; 84.0%). Denuded presumptive zygotes at the pronuclear stage (14–18 h post-insemination) were assigned randomly to the same four treatment groups and, following thawing, embryos were assessed for their capacity to cleave and to develop into a blastocyst. Overall, cleavage rates of cryopreserved zygotes were significantly less than those of the fresh control. The blastocyst formation rate of slow-frozen zygotes (4/81; 4.9%) was significantly less than that of zygotes subjected either to short equilibration vitrification (18/82; 22.0%) or long equilibration vitrification (16/74; 21.6%). All cryopreservation groups showed rates of blastocyst formation that were significantly less than that of the fresh control (51/92; 55.4%). Collectively, our findings indicate that vitrification is the preferred technology to cryopreserve *in vitro*-derived cattle embryos at expanded blastocyst and pronuclear stages. Moreover, short equilibration vitrification technology can improve outcomes and be more efficient by taking less time to perform.

1. Introduction

Cryopreservation plays an essential role in the commercial application of assisted reproductive technologies in cattle. Yet, while conventional slow-rate freezing is considered routine for *in vivo*-derived embryos [20], with a feasible direct transfer method using ethylene or propylene glycol as cryoprotectants [12], there are substantial problems with this method of freezing for use with *in vitro*-derived cattle embryos [33]. Embryos derived *in vitro* have a lesser survival capacity than do *in vivo*-derived embryos [5], which leads to the limited application of *in vitro* fertilization technologies in commercial enterprises [4]. Vitrification is an alternative cryopreservation method to slow freezing that has shown improved clinical outcomes for human oocytes and embryos [18]. Currently, however, considerable variation exists between vitrification procedures [9], and each species or embryo type requires particular refinements so as to optimize vitrification efficiency. While Sanches et al. [26] have reported promising outcomes for *in vitro*-derived cattle embryos, further refinements are required in order to ensure a robust system [11,33].

In the present study, conventional slow freezing was compared with two vitrification protocols: long equilibration vitrification

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(performed at room temperature); and short equilibration vitrification (performed at 37 °C), with the objective of identifying an improved cryopreservation protocol for the storage of *in vitro*-derived cattle embryos [11]. The expanded blastocyst stage of development was chosen to assess this approach.

If *in vitro* culture conditions are not ideal it may also be useful to cryopreserve embryos earlier during development, at the pronuclear stage, with the potential for *in vitro* development or transfer of either the pronuclear or cleavage stage after thawing. Vajta et al. [34] reported cryopreservation of cattle oocytes that had been matured for either 6 h or 22 h and of cattle embryos that had been collected from d 1–7 (d 0 = day of fertilization); however, it is unclear if the d 1 embryos used in this study were all at the 1-cell pronuclear stage. To our knowledge, the cryopreservation of *in vitro*-derived pronuclear stage cattle embryos is seldom reported, although the success of pronuclear cryopreservation has been documented for some mammalian species including mice [3,8] and humans [2,19,27].

It is important to note that identification of pronuclear cattle embryos is a difficult task, even for an experienced bovine embryologist. Extrusion of the second polar body, indicative of successful fertilization, is not always clearly visible, and while in mice and humans the two pronuclei are easily observed under a dissecting microscope this is not possible with cattle embryos due to their greater relative lipid content. Following fixation and staining of presumptive early zygotes, an interval of 16–28 h post-insemination was considered to be the period during when the late pronuclear stage of zygote development occurs [35].

With the routine use of *in vitro* fertilization (IVF) systems in the laboratory where the present research was conducted, it has been observed often that after 18 h several two-cell embryos may develop. In the current investigation, therefore, presumptive zygotes, denuded of surrounding cumulus cells, were collected 14–18 h post-insemination for an assessment of *in vitro*-derived cattle embryos at pronuclear stages after cryopreservation. The three freezing protocols examined previously were followed to cryopreserve presumptive cattle zygotes and the capacity of these embryos to tolerate freezing using the three methods was compared.

The overarching objective of the present experiments was, therefore, to compare the application of three distinct cryopreservation procedures to the preservation of *in vitro*-derived cattle embryos at the expanded blastocyst and pronuclear stages and to investigate if a universal protocol may be used for all stages of development.

2. Materials and methods

2.1. Experimental design

Two *in vitro* experiments were designed to evaluate the efficacy and efficiency of three cryopreservation protocols on the survival of *in vitro*-derived cattle embryos at expanded blastocyst and pronuclear stages of development. All standard chemicals and reagents were purchased from Sigma-Aldrich (Melbourne, VIC, Australia) unless otherwise stated.

2.1.1. Experiment 1. Effect of different cryopreservation protocols on survival and hatching capacity of expanded blastocysts

In vitro-derived, expanded blastocysts were assigned randomly to four treatment groups (fresh control, slow freezing, short equilibration vitrification and long equilibration vitrification). Procedures conducted depended on the number of expanded blastocysts obtained at the time of embryo collection. For *in vitro* procedures to be performed required at least three expanded blastocysts, which were assigned randomly to treatment groups. These blastocysts were frozen, vitrified or cultured unfrozen (fresh control group). After a minimum of 7 d, embryos were warmed or thawed and expansion of the blastocyst after 24 h in *in vitro* culture was determined. The number of blastocysts that hatched during a 48 h culture period was also recorded.

2.1.2. Experiment 2. Effects of different cryopreservation protocols on rates of cleavage and blastocyst development from presumptive zygotes at pronuclear stages

Cumulus oocytes complexes (COCs) were assigned randomly to *in vitro* maturation groups. Oocytes were fertilized *in vitro* and at 14–18 h post-insemination were frozen or vitrified. Presumptive zygotes were examined, all were observed to degenerate and immature oocytes (assessed as having no visible polar body) were removed. The remaining presumptive zygotes were assigned randomly to the four treatment groups (fresh control, slow freezing, short equilibration vitrification and long equilibration vitrification). After at least 7 d of storage, presumptive zygotes were warmed or thawed. Presumptive zygotes were subsequently cultured for 5 d. On d 5 of culture, the percentages of embryo cleavage for the different groups were recorded. On d 7 of culture, blastocysts were collected and examined. Non-cryopreserved controls were cultured unfrozen as described above.

2.2. Production of in vitro-derived cattle embryos

2.2.1. In vitro maturation (IVM) of oocytes

Ovaries from beef cattle were collected from a local abattoir and delivered to the laboratory within 1 h in a flask containing sterile-filtered saline (0.9% NaCl; Baxter Healthcare, Old Toongabbie, NSW, Australia) at 30–35 °C. Fluid was aspirated from follicles and placed in a petri dish (Falcon®; Corning Life Sciences, Clayton, VIC, Australia) to search for oocytes. Oocytes surrounded completely with several layers of tight COCs were chosen and washed twice in 100 μL VitroWash® medium (IVF VET Solutions, Adelaide, SA, Australia). In repeat procedures, between 25 and 30 COCs were co-cultured in 150 μL IVM medium (VitroMat®, IVF VET Solutions) for 22–24 h in an incubator set to 38.8 °C and supplied with 5% O_2, 5% CO_2 and 90% N_2.

2.2.2. In vitro fertilization (IVF) of oocytes

For each IVF procedure, a straw of sperm obtained from a bull of proven fertility was used. The straw was taken from liquid N_2 storage and held in the air for 10 s, then submerged into a 37 °C water bath for at least 30 s. After cutting the straw, semen was placed in a tube containing a discontinuous 45:90% density gradient (BoviPure™, Nidacon, Mölndal, Sweden) that had been pre-warmed to 38.5 °C and spun at 1500 × *g* for 15 min without braking. Following centrifugation, the supernatant was removed and 500 μL BoviWash™ medium (Nidacon) was added slowly in order to gently resuspend the sperm pellet. This was centrifuged a second time at 1500 × *g* for 5 min without braking and again the supernatant was removed. The concentration of sperm was adjusted to 1×10^6 sperm/mL with IVF medium (VitroFert®, IVF Vet Solutions) supplemented with 4 mg/ml bovine serum albumin (BSA), 10 IU/ml heparin, 25 μM penicillamine, 12.5 μM hypotaurine and 1.25 μM epinephrine.

Meanwhile, COCs were washed once in 100 μL VitroFert® medium, resuspended in 300 μL VitroFert® medium and overlaid with 3.5 ml mineral oil. The sterile Nunc™ IVF polystyrene petri dish (Thermo Fischer Scientific, Scores by VIC, Australia) was incubated at 38.8 °C in 5% O_2, 5% CO_2 and 90% N_2.