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Effect of cryopreservation technique and season on the survival of *in vitro* produced cattle embryos

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ABSTRACT

Embryo cryopreservation is a major tool for conservation and propagation of genetically superior animals. However, it adversely affects the survival of embryos. The objective of this study was to determine the effects of cryopreservation technique (vitrification compared with slow freezing) and different seasons in which oocytes were obtained on the post-warming survival of *in vitro* produced (IVP) cattle morulae. In experiment 1, morulae (Day 6 post-IVF), obtained from abattoir-sourced oocytes during spring, summer, fall and winter over a period of 3.5 years, were subjected to either vitrification ($n = 271$ morulae), slow freezing ($n = 281$ morulae) or no freezing (control; $n = 249$ morulae). After warming, the morulae were cultured to the expanded blastocyst stage (Day 8 post-IVF). Data were compared using Glimmix procedure in SAS®. Blastocyst rate differed ($P < 0.05$) among the treatments: unfrozen control ($78 \pm 3.6\%$), vitrification ($52 \pm 4.6\%$) and slow freezing ($35 \pm 4.2\%$). The re-expansion of vitrified morulae upon warming was not correlated with subsequent blastocyst rate ($r = -0.048$; $P > 0.05$). The morulae produced during fall season had lesser ($P < 0.05$) cleavage and morula rates ($67 \pm 1.6\%$; Day 2 post-IVF and $22 \pm 1.4\%$; Day 6 post-IVF, respectively) than all other seasons (74 ± 1.1 and $30 \pm 1.2\%$, respectively). Blastocyst rate was the least ($P < 0.05$) when oocytes were collected during the summer season in both control and slowly frozen groups. Blastocyst development rate did not change due to season in vitrification group ($P > 0.05$). In conclusion, vitrification is a more desirable technique than slow freezing for cryopreservation of IVP cattle morulae. If the slow freezing method is employed, greater success can be achieved using oocytes collected in the winter and spring with a primary contributing factor being lesser morulae development if oocytes are collected in the fall and also the lesser blastocyst formation of cryopreserved morulae when oocytes are collected in the summer.

1. Introduction

Embryo cryopreservation is an important part of embryo transfer industry. Several hundred thousand frozen cattle embryos produced *in vivo* and *in vitro* have been exported from one country to another for purposes of subsequent transfer. Similar to other cells, conventional freezing damages mammalian embryos due to intracellular

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ice formation and toxic effects of permeating cryoprotectants. Moreover, embryo cryopreservation is one of the strategic approaches to conserve and improve the genetic potential of a breed. Cryobiologists aim to minimize the damage caused by intracellular ice crystal formation during the freezing and thawing cycle by using techniques that reduce this problem. The intracellular ice formation is dependent on cooling rate and surface area/volume of cells (Mazur, 1963, 1970). Cattle embryos are conventionally frozen using a programmed slow freezing procedure. A simple and ultra-rapid freezing technique "vitrification" has been introduced (Vajta and Nagy, 2006). Both techniques are differentiated by two factors: cryoprotectant concentration and cooling rate (Leibo and Loskutoff, 1993; Massip, 2001). The slow cooling rate, in conventional freezing, increases extracellular osmolality due to ice formation that leads to an efflux of intracellular water and cell dehydration (shrinkage). Concomitantly, the solute concentration in the cytoplasm increases which decreases the intracellular freezing temperature. Cells continue to dehydrate and shrink with extracellular ice formation and the cytoplasm undergoes vitrification. In rapid cooling, the cells do not dehydrate completely, and intracellular water forms ice crystals (Mazur, 1963, 1970). Vitrification, however, is based on the preservation of cells and tissues in a highly viscous solution of cryoprotectants to achieve a glass-like state with ultra-rapid cooling rates (>5000 °C/min) thus avoiding intracellular ice formation (Rall and Fahy, 1985; Rall, 1987; Kuwayama, 2007).

In vitrification, embryos also undergo severe dehydration (shrinkage) due to greater sucrose (non-permeating cryoprotectant) concentrations. During warming, the vitrified embryos rehydrate and regain their shape and volume. In a study with 8- to 16-cell mouse embryos, the impact of method of cryoprotectant equilibration on embryo re-expansion (volume change) and post-thaw blastocyst development was observed (Szell and Shelton, 1987). This "embryo re-expansion" is considered an indicator of embryonic viability and developmental potential (Shaw et al., 1991; Tachikawa et al., 1993; Kaidi et al., 2000; Aksu et al., 2012). A secondary objective of the current study was to validate the relationship between re-expansion of vitrified cattle morulae upon warming and subsequent blastocyst formation.

Oocyte quality and embryo survival in lactating dairy cattle are adversely affected by increased summer ambient temperatures (Gendelman et al., 2010; Braga et al., 2012; Gendelman and Roth, 2012). *In vitro* fertilization (IVF) has been used as an alternative to circumvent the detrimental effects of thermal stress on fertility (Rutledge, 2001; Al-Katanani et al., 2002). However, the impact of season in which the cattle embryos are produced *in vitro* on embryo survival with cryopreservation has not been explored extensively. Accordingly, the primary objectives of the present study were to determine the effects of common cryopreservation techniques (slow freezing compared with vitrification) and season of year (spring, summer, fall and winter) on the survival of *in vitro* produced cattle embryos.

2. Material and methods

The procedures used in this study were approved by the Animal Care Committee and Animal Research Ethics Board, University of Saskatchewan, Saskatoon, Canada.

2.1. Chemicals and supplies

Dulbecco's phosphate buffer saline (DPBS-1X Ca^{2+}-Mg^{2+} plus- Cat# 21300-025), Newborn calf serum (CS), TCM-199 (Cat# 12340-030) and MEM essential amino acids (Cat# 11140-050) were purchased from Invitrogen® Inc. (Burlington, ON, Canada). Lutropin-V (LH), Folltropin-V (FSH; Cat # PHD075) and Vigro™ Ethylene Glycol Freeze Plus media (Cat#624034) were obtained from Bioniche® Animal Health, Inc. (Belleville, ON, Canada). All other chemicals and reagents were purchased from Sigma–Aldrich® (Oakville, ON, Canada). Cryotops (Cat# 81111) used in vitrification were purchased from Kitazato® Co. (Fuzi, Shizuoka, Japan). Mini-straws (0.25 mL) used in slow freezing were purchased from IMV® Tech., (Woodstock, ON, Canada).

2.2. In vitro embryo production

Ovaries of cattle were procured from a commercial abattoir near Calgary (51°N, 114°W) during four different seasons (spring, summer, fall and winter) and were transported to the Cryobiology Laboratory (joint initiative of Agriculture and Agri-Food Canada and Western College of Veterinary Medicine), Saskatoon (52°N, 107°W) Canada, in a controlled temperature cooler at 22 °C within 12 h. All subsequent laboratory procedures (oocyte aspiration, *in vitro* maturation, *in vitro* fertilization and embryo culture) were completed within a week from the time of collection in the same season of the year. Other tissues were carefully removed and ovaries were washed with physiological saline. *In vitro* maturation (IVM) and fertilization (IVF) and embryo culture were conducted as previously described (Prentice et al., 2011). Briefly, cumulus oocyte complexes (COC) were aspirated from 2 to 8 mm follicles using an 18-gauge needle attached to a 3-mL syringe containing 1 mL holding solution [HS; 5% (v/v) new born calf serum (CS) in DPBS]. The COC were identified in the aspirated (pooled) follicular fluid under a stereomicroscope, washed (3×) in HS and graded (de Loos et al., 1989). The COC (Grade 1 and 2), with more than three layers of cumulus cells, a uniform cytoplasm and no visual signs of degeneration were selected for further processing. The COC were washed (3×) in maturation medium containing TCM-199, 5% (v/v) CS, 5 μg/mL LH, 0.5 μg/mL FSH and 0.05 μg/mL gentamicin at 37 °C. The groups of 20 COC were placed in 100-μL droplets of maturation medium under mineral oil and incubated at 37 °C in 5% CO_2 and high humidity, for 22 to 24 h.

Frozen semen straws from Holstein dairy bulls were thawed in a water bath at 37 °C, for at least 1 min. Sperm were washed and live sperm were harvested using the Percoll density gradient method (Parrish et al., 1995). The sperm concentration was adjusted to 3×10^6 cells/mL in Brackett-Oliphant fertilization medium (Brackett and Oliphant, 1975). Eight 100-μL droplets of diluted sperm