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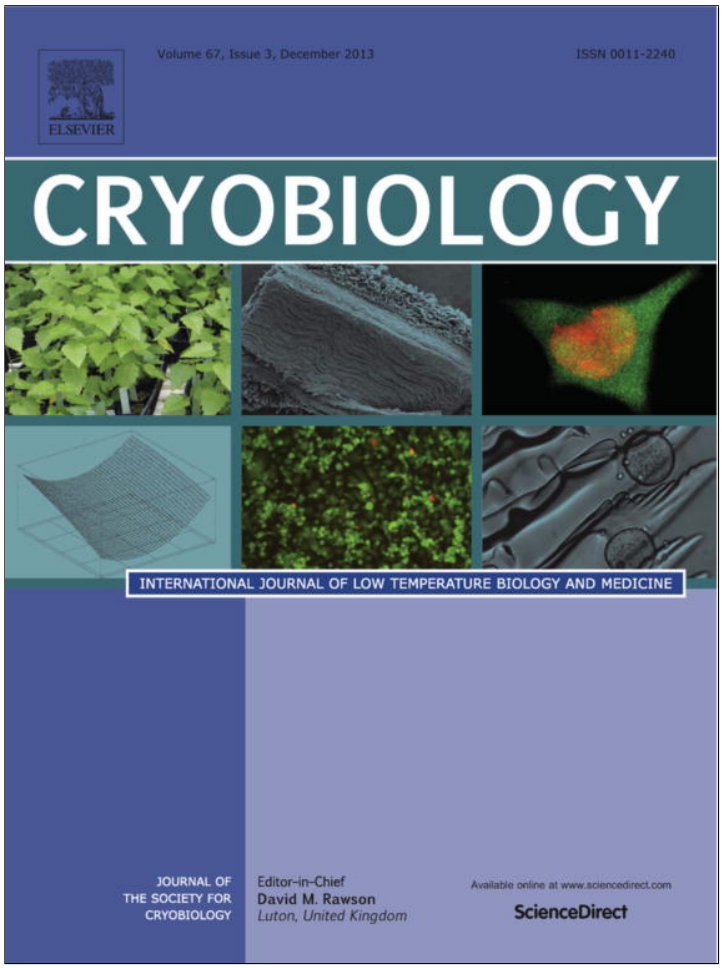

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Brief Communication

Evaluation of the Cryotech Vitrification Kit for bovine embryos ☆

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ABSTRACT

The purpose of this work was to assess commercially available Cryotech Vitrification Kit, in terms of survival, *in vitro* development and pregnancy rate for bovine embryos. Cumulus-oocyte complexes (COCs) were recovered from ovaries obtained from slaughtered cows and then matured *in vitro* for 22 h. COCs were fertilized by sex-sorted sperm in IVF-mSOF and cultured in IVC-mSOF for 7 days to the blastocyst stage. Blastocysts were vitrified with the Cryotech Vitrification Kit® and then either warmed to check viability or transferred to synchronized heifers. We observed 100% survival of the *in vitro* produced blastocysts and obtained the same pregnancy rate (46.8%) as that obtained using fresh *in vitro* produced blastocysts. We thus conclude that the Cryotech vitrification method is a valid alternative to other vitrification or slow-cooling methods in the bovine species and that it is ready for livestock production.
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Introduction

Although cryopreservation of bovine embryos is a well known technique that has been commercially widely used, it can be difficult to know which cryopreservation method to use. Slow programmable freezing, a standard method for bovine embryo cryopreservation, achieves embryo survival rates of between 40% and 60% [1]. Although vitrification has become an alternative for cryopreservation of mammalian embryos [2], it is still not used routinely in the bovine species as current vitrification methods lead to highly variable results [3–6]. The aim of this work was to evaluate the Cryotech Vitrification Method on the embryo viability and pregnancy rates of *in vitro* produced bovine embryos.

Materials and methods

Ovaries from Holstein cows of high genetic quality were obtained from an abattoir within 30 min of slaughter and kept warm (30 °C) during the 2-h journey to the laboratory. Ovaries were washed in physiologic saline containing 100,000 U penicillin L^{-1} and 100 mg streptomycin L^{-1}. Cumulus oocyte complexes (COCs) were recovered by aspiration of antral follicles (2–5 mm in diameter) and only oocytes completely surrounded by compact and multilayered cumulus cells were used. Groups of 50 COCs were matured in 500 μL medium 199 (Earle's salts, L-glutamine, sodium bicarbonate 2.2 mg L^{-1}, GIBCO, Grand Island, NY, USA) supplemented with 5% (v/v) fetal bovine serum (FBS, GIBCO), 0.2 mg porcine FSH L^{-1} (Folltropin-V, Bioniche, Belleville, Ontario, Canada), 2 mg porcine LH L^{-1} (Lutropin-V, Bioniche) and 50 mg gentamicin sulfate L^{-1} under mineral oil at 39 °C for 22 h in an atmosphere of humidified CO_2 (5%) in air.

In vitro fertilization was performed using sex-sorted frozen-thawed semen of a Holstein bull of proven fertility. Semen was thawed at 37 °C in modified synthetic oviductal fluid (mSOF) [7], centrifuged at 500×*g* twice for 5 min and then resuspended in fertilization medium to a final concentration of 2×10^6 motile spermatozoa L^{-1}. Fertilization was performed in 500 μL IVF-mSOF containing mature oocytes and sperm, consisting of mSOF added with 6 g bovine serum albumin (BSA) L^{-1}, 10,000 U heparin L^{-1}, under mineral oil at 39 °C, 5% CO_2 in air and 100% humidity for 20 h. Putative zygotes were then denuded by repeated pipetting and placed in 500 μL IVC-mSOF, consisting of mSOF supplemented with 30 mL amino acid MEM L^{-1} (GIBCO), 10 mL non-essential amino acid MEM L^{-1} (GIBCO), 2 mmol L-glutamine L^{-1}, 6 g BSA L^{-1} and 5% (v/v) FBS (GIBCO), under mineral oil at 39 °C, 90% N_2:5% CO_2:5% O_2 and 100% humidity. The proportion of cleaved oocytes was determined at 48 h of sperm co-culture by evaluating the number of embryos that presented two or more blastomeres. An additional cohort of 10 oocytes for each replicate was maintained through the fertilization procedure without exposure to sperm to test parthenogenesis. This resulted in 0% of cleavage. *In vitro* embryo development was performed in IVC-mSOF medium [7], under mineral oil at 39 °C, 90% N_2:5% CO_2:5% O_2 and 100% humidity, renewing the culture medium every 48 h up to blastocyst stage on day 7 following insemination.

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Vitrification

All the embryos were vitrified and warmed by three different embryologists by using the Cryotech Vitrification Method® (Repro-Support Medical Research Centre, Co. Ltd., 2-5-5-8F Shinjuku, Tokyo, Japan). The key point of this vitrification method is to base the exposure times on the reaction of embryo, as detected by the eyes of the embryologist, rather than the absolute time taken in the process. Briefly, embryos were equilibrated at room temperature in the solution supplied with the kit containing ethyleneglycol (EG) and dimethylsulphoxide (Me_2SO) in MEM with hydroxypropyl cellulose (HPC) and no protein supplement. The embryos shrank and then recovered their normal morphology. This process took between 10 and 15 min depending on the quality of each embryo. Then, the embryos were moved to a vitrification solution at room temperature containing EG + Me_2SO, and trehalose in MEM + HPC until the embryo shrank sharply again and sunk in the vitrification media. The embryos were then loaded individually with a glass capillary onto the top of the film strip supplied with the kit, with a minimal amount of liquid and the sample was quickly immersed into liquid nitrogen and covered with a protective cap. The time between the immersion into the vitrification solution and the immersion into the liquid nitrogen was 60–90 s. At warming, the strip was immersed directly into warming solution consisting of MEM + trehalose + HPC at 37 °C for 1 min. The embryos were incubated for 3 min in the kits diluent solution consisting of MEM + trehalose + HPC and washed twice for 5 min in washing solutions consisting of MEM + HPC.

After warming, embryos were cultured in IVC-mSOF medium for 3 h and embryo viability was then evaluated by morphology and fluorescein diacetate (FDA) staining according to the method previously described by Mohr and Trounson [8]. Briefly, embryos were treated with 0.25 μg% FDA in phosphate buffer saline (PBS) (m/v) supplemented with 1% polyvinyl alcohol (PVA) (m/v) at 37 °C for 15 min in a dark room and then washed three times in PBS supplemented with PVA and evaluated under a fluorescent microscope with UV irradiation, using a filter with an excitation wavelength of 460–495 nm and emission of 510 nm. Embryos showing a bright green fluorescence were regarded as living ones.

Embryo transfers

At the beginning of the synchronization program, *Bos taurus* heifers were examined by transrectal ultrasonography to determine cyclicity (evidence of luteal tissue) and those with abnormalities in the reproductive tract were eliminated. On Day 0, heifers at random stages of the estrous cycle received a controlled internal drug release (CIDR; Bioniche Animal Health Canada Inc., Belleville, ON, Canada) vaginal insert and 1 mg estradiol cypionate i.m. (ECP; Pharmacia Animal Health, Orangeville, ON, Canada). The CIDRs were inserted into the vagina and on Day 8, they were removed and heifers received 25 mg dinoprost (Lutalyse; Pharmacia Animal Health). Twenty-four hours later (day 9), heifers received 0.5 mg ECP i.m. Day 10 was arbitrarily considered as the day of estrus and on day 17 one fresh or one vitrified blastocyst was transferred to each heifer. Transrectal ultrasonography for pregnancy diagnosis was done 35 days after embryo transfer.

Blastocysts for transfer were all generated from oocytes derived from ovaries from slaughtered cows which were matured, fertilized and cultured *in vitro* up to blastocyst stage in three different trials. This resulted in a pool of 161 blastocysts. Fifteen of fresh blastocysts were transferred immediately to synchronized recipient heifers as controls.

Results

A total of 50 vitrified embryos were used to observe their morphology and viability after 3 h of culture. All these blastocysts showed normal morphology: recovery of blastocoel and well-defined blastomere membranes. All the blastocysts stained with FDA solution disclosed a bright green fluorescence (viability = 100%). Seven of the fifteen recipients of fresh blastocysts became pregnant (46.6%), and four healthy calves (26.6%) (two females and two males) were born.

The remaining vitrified *in vitro* produced blastocysts ($n = 96$) were warmed by three different operators in four different farms, loaded in individual straws and directly transferred to synchronized recipient heifers. A total of 45 pregnancies (46.8%) were confirmed and 33 healthy calves (34.3%) (28 females (84.8%) and 5 (15.2%) males) were born.

This study was carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

Discussion

The purpose of this work was to assess Cryotech Vitrification Kit®, in terms of survival, *in vitro* development and pregnancy rate of bovine embryos. We can report a highly efficient, low-cost, easy-to-perform and easy-to-learn vitrification method for cattle embryos. In addition, the method was consistent among operators and trials, thus making the results more trustworthy. Studies carried out during the last three years have reported viability results of other vitrification methods ranging from 27 to 87% [3–6]. The vitrification system used in this work allowed us to obtain 100% of survival of *in vitro* produced blastocysts assessed by morphological and fluorescence viability assays. Furthermore, it yielded the same pregnancy rates as those obtained using fresh IVP blastocysts in our control and were comparable to those previously reported in the literature [9,10].

The Cryotech vitrification Method has therefore proved to be flexible, and can be used with embryos at optimal developmental stage when it is convenient for the lab staff, with no fear of malfunction, consistency in performance. In addition, it ends in a few minutes with 100% success. Although this vitrification has become widely used in human reproduction, it is still not used routinely in domestic animals despite the impact of the survival results and pregnancy rates obtained [11]. During the last 20 years, different research groups have dedicated a large amount of work to the improvement of the vitrification protocols of bovine embryos and have shown that vitrification provides good results when bovine embryos are cryopreserved. We thus conclude that the Cryotech Vitrification Method is a valid alternative to vitrification or slow-cooling methods in the bovine species and that it is ready for livestock production.

With this low cost and easy-to-perform vitrification method for bovine embryos, amazing rates of survival (up to 99%) can be obtained. The extended use of this method could result in great advantages for the field of domestic animal embryology and reproduction.

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