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Ice nucleating agents allow embryo freezing without manual seeding

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ABSTRACT

Embryo slow freezing protocols include a nucleation induction step called manual seeding. This step is time consuming, manipulator dependent and hard to standardize. It requires access to samples, which is not always possible within the configuration of systems, such as differential scanning calorimeters or cryomicroscopes. Ice nucleation can be induced by other methods, e.g., by the use of ice nucleating agents. Snomax is a commercial preparation of inactivated proteins extracted from *Pseudomonas syringae*. The aim of our study was to investigate if Snomax can be an alternative to manual seeding in the slow freezing of mouse embryos. The influence of Snomax on the pH and osmolality of the freezing medium was evaluated. *In vitro* development (blastocyst formation and hatching rates) of fresh embryos exposed to Snomax and embryo cryopreserved with and without Snomax was assessed. The mitochondrial activity of frozen-thawed blastocysts was assessed by JC-1 fluorescent staining. Snomax didn't alter the physicochemical properties of the freezing medium, and did not affect embryo development of fresh embryos. After cryopreservation, the substitution of manual seeding by the ice nucleating agent (INA) Snomax did not affect embryo development or embryo mitochondrial activity. In conclusion, Snomax seems to be an effective ice nucleating agent for the slow freezing of mouse embryos. Snomax can also be a valuable alternative to manual seeding in research protocols in which manual seeding cannot be performed (i.e., differential scanning calorimetry and cryomicroscopy).

1. Introduction

The aim of embryo cryopreservation is to ensure high survival rates and high viability rates after long periods of storage [1]. There are two main strategies to cryopreserve embryos: slow freezing and vitrification. During the slow freezing procedure, extracellular ice crystal formation occurs and embryos survive in a glassy state between ice crystals [2] while in vitrification all of the solution turns into a glass-like state without ice crystal formation [3].

In slow freezing, the extracellular crystallization is a crucial event, since it increases the concentration of the extracellular liquid, contributing to cell dehydration, and preventing the formation of intracellular ice crystals. During slow freezing, the temperature of the system is lowered gradually in a controlled manner. Water molecules can cool below their melting temperature (0 °C) but remain in an unstable liquid state through a process called supercooling, until the nucleation process occurs and the crystallization phenomenon starts. Without external perturbation, solutions commonly supercool (remain liquid) to more than 10 °C below the maximal melting temperature [4].

The mechanical damages caused by the formation of ice crystals or ice front displacement are an important cause of cellular death during slow freezing. The growth and structure of the ice crystals depend on the supercooling magnitude [5]. Moreover, the probability of intracellular ice formation increases with the degree of supercooling [6,7]. For this reason, control of the nucleation temperature is crucial to prevent this damaging process.

To minimize the supercooling magnitude, automatic or manual induction of the nucleation (manual seeding) need to be performed [8]. Some programmable freezers include an option to induce automatic seeding. However, this is not routinely used in embryo freezing, since it was shown to have a deleterious effect on the embryo survival rate and development rate [9]. Manual seeding is routinely performed by cooling a spot on the outside of the straw, with a cold instrument, which locally decreases the temperature of

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the sample and induces nucleation [4,5]. However, this step presents several drawbacks. When performed inside of a programmable freezer, manual seeding requires the manipulator's presence and rapid access to the samples through the opening of the freezer. It is also time-consuming, and it is difficult to seed more than one sample at a time. Moreover, opening the programmable freezer may result in a non-controlled temperature rise [5], compromising the standardization of this step. When a strictly standardized slow freezing protocol is needed, in particular in thermodynamic studies, an alternative to manual seeding is essential.

Some thermodynamic studies may require embryo slow freezing inside of other system configurations, such as differential scanning calorimetry or cryomicroscopy. In these situations, access to the samples during the protocol is not possible, so manual seeding cannot be applied. To study embryo slow freezing inside of these equipment, a manual seeding substitute is imperative.

Whether one's goal is to simplify and better standardize embryo slow freezing in programmable freezers or to simulate manual seeding in equipment where access to the samples is not possible, a reliable alternative to this mandatory step is missing.

To induce nucleation at higher temperatures, ice nucleating agents (INA) can be added to the freezing media [4,6]. They facilitate the orientation of water molecules in an ice-like structure to create active germ crystals. This effect promotes the freezing of supercooled water at temperatures higher than expected [10]. Among the numerous substances that can serve as ice nuclei, we find: inorganic crystalline solids, such as silver iodide [11], successfully used to induce seeding in bovine and rabbit embryos [12,13]; substances present in atmospheric aerosols, such as mineral dusts or metallic particles [14]; and biological ice nucleating agents, which are produced by multiple organisms. The most frequently studied ice nucleating agents of biological origin are substances present in some bacteria membranes (i.e., *Pseudomonas syringae, P. viridiflava, P. fluorescens, Pantoea agglomerans* and *Xanthomonas campestris*). These bacteria are usually found in association with plants and are capable of inducing ice nucleation at temperatures slightly below 0 °C [11,15].

Snomax (Snowmakers AG, Steffisburg, Switzerland) is a commercial preparation of freeze-dried inactivated proteins extracted from *P. syringae*, sterilized by gamma irradiation. It is used as an additive to produce artificial snow. This commercial preparation has been used in differential scanning calorimetry physical experiments [14,15,18], cryomicroscopy [6,7,17] and food industry experiments [16]. In 2015, our research team demonstrated that Snomax can be a valuable tool to control, in a replicable manner, the crystallization temperature of a solution [17].

The aim of our study was to validate the use of Snomax as a manual seeding substitute in embryo slow freezing by evaluating the physiological characteristics of the slow freezing medium and the impact of this ice nucleating agent on the development of fresh and frozen-thawed embryos.

2. Materials and methods

The Ethical and Animal Welfare Committee of VetAgro Sup approved this study (agreement number 1307). All animals were handled according to the EU Directive 2010/63/EU for animal experiment guidelines.

Unless specified otherwise, all of the chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.1. Embryo cryopreservation media composition

Two slow freezing media were used. The slow freezing medium of the “manual seeding group” and of the “no-seeding group” contained 1.5 M DMSO in Embryo holding medium (IMV Technologies, L'Aigle, France) and the slow freezing medium of the “Snomax group” contained 1.5 M DMSO in Embryo holding medium supplemented with Snomax (10 mg/L). Snomax concentration was selected after performing previous thermodynamic (unpublished) studies, were the authors observed a reproducible crystallization temperature of approx. −7 °C. Other studies using Snomax as oocyte nucleation inductor describe the same Snomax concentration [6,7,18,19].

2.2. Evaluation of osmolality and pH

The osmolality and pH of the two cryopreservation media were measured at room temperature, using a Vapro 5600 vapour pressure osmometer (Logan, USA) and a Mettler Toledo FE20 pH Meter (Schwerzenbach, Switzerland), respectively.

2.3. Embryo production and recovery

A total of 42 outbred RjOrl: Swiss mature mice were used in this study: 32 females and 10 males. Females were housed in groups of up to 6 and males were housed individually, under a controlled 14 h light/10 h dark photoperiod and fed with a commercial diet.

Female mice were submitted to a superovulation treatment by administrating 10 IU of pregnant mare's serum gonadotrophin (PMSG) and, 48 h later, 10 IU of human chorionic gonadotrophin (hCG) subcutaneously. Four hours after the last administration, each female was placed with one male and mating was confirmed by the presence of a vaginal plug the next morning.

The oviducts and uteri of mated females were flushed (room temperature) 68–72 h after the hCG administration. The flushing medium was composed of Dulbecco's Phosphate-Buffered Saline medium (DPBS) supplemented with D-glucose, sodium pyruvate and bovine serum albumin (BSA). Embryos (8-cell to early compacting morula stages) were classified according to the International Embryo Transfer Society manual [20]. Fair and good quality embryos were pooled and randomly divided into different study groups.

2.4. Snomax's effect on development

Snomax's effects on development were evaluated by assessing the embryo development potential after incubating fresh embryos in a solution containing Snomax. In this experiment, none of the groups contained cryoprotectants.

Briefly, a total of 52 embryos were divided into 3 groups and incubated in the corresponding incubation medium for 45 min. Two control groups were incubated: one in the “culture medium” composed of M16 (37 °C, 5% CO_2), and one in a “freezing based medium” composed of Embryo holding medium (37 °C, atmospheric air). The third group was incubated in a “Snomax medium” (37 °C, atmospheric air) composed of Embryo holding medium supplemented with Snomax (10 mg/mL).

After incubation, the embryos were washed in culture medium and cultured to the hatching stage.

2.5. Embryo culture and morphology assessment

Embryos were cultured (37 °C, 5% CO_2) in M16 medium supplemented with antibiotics (penicillin 67 μg/mL and streptomycin 67 UI/ml, Dutscher, Brumath, France), under paraffin oil (Ovoil, Vitrolife, Göteborg, Sweden).

In vitro development was assessed at day 1, 2 and 3 of culture. Delayed embryos and embryos classified as poor or degenerating embryos were eliminated.