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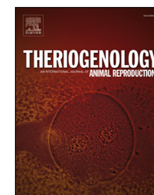

Noninvasive embryo assessment technique based on buoyancy and its association with embryo survival after cryopreservation

Cara Wessels [a,*], Lindsay Penrose [b], Khaliq Ahmad [b], Samuel Prien [a,b]

[a] Texas Tech University, 2500 Broadway, Lubbock, TX 79409, United States
[b] Texas Tech University Health Sciences Center, 3601 4th St, Lubbock, TX 79430, United States

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ABSTRACT

Embryo cryopreservation offers many benefits by allowing genetic preservation, genetic screening, cost reduction, global embryo transport and single embryo transfer. However, freezing of embryos decreases embryo viability, as intracellular ice crystal formation often damages embryos. Success rates of frozen embryo transfer are expected to be 15–20% less than fresh embryo transfer. We have developed a noninvasive embryo assessment technique (NEAT) which enables us to predict embryo viability based on buoyancy. The purpose of this research was twofold. First was to determine if a NEAT, through a specific gravity device can detect embryo survival of cryopreservation. Second, it was to relate embryo buoyancy to embryo viability for establishing pregnancies in sheep. Blastocysts descent times were measured on one-hundred sixty-nine mice blastocysts before cryopreservation, according to standard protocol and post-thawing blastocysts descent times were measured again. There was a significant difference in blastocyst post-thaw descent times with NEAT in those blastocysts which demonstrated viability from those that did not ($P < 0.05$). This suggests NEAT is successful in determining blastocysts viability in cryopreserved mice blastocysts. At a commercial ovine facility, NEAT was performed on fourteen frozen and thawed ovine blastocysts. Blastocysts of similar descent times were paired and transferred into recipient ewes as twins. Pregnancy was later confirmed by blood test and multiple gestation outcomes were determined at lambing. Six of seven recipient ewes were pregnant and all pregnant ewes delivered lambs without complication. Four ewes delivered twin lambs and two ewes delivered singletons, which totals ten of the fourteen (71%) blastocysts surviving to term. This pregnancy rate is comparable to expected to pregnancy rates in a commercial setting. The blastocysts which did not establish pregnancy demonstrated less buoyancy versus those blastocysts which established pregnancies which survived to term ($P < 0.05$). These results suggest NEAT can identify which blastocysts survive cryopreservation, thus significantly reduce the transfer of non-viable embryos. Further studies on a larger scale commercial setting will evaluate the efficacy of NEAT.

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1. Introduction

Cryopreservation, or storage of viable cells at low temperatures in liquid nitrogen, has improved Advanced Reproductive Technologies (ARTs) by simplifying the embryo transfer process, allowing genetics to be preserved over time, promote single embryo transfer, reduce cost, allow superior genetics to be shipped globally and promote the use of preimplantation genetic screening. Since the first birth of a frozen mouse embryo in 1971, cryopreservation has become a mainstream procedure with over 500,000 bovine frozen-thawed embryos transferred annually [1,2,3]. However, cryopreservation does not guarantee embryo viability, as the technique can induce cryodamage due to the formation of intracellular ice crystals. Youngs reports cryopreserved embryo transfer success rates to typically be at least 20% lower than seen with fresh embryos [4]. It is generally believed such decreases are the result of cryodamage to the embryos during storage. However, there is currently no method to detect embryo survival of cryopreservation beyond simple morphological assessment.

Previous research from our laboratory has suggested a noninvasive embryo assessment technique (NEAT) can be used to

* Corresponding author.
E-mail addresses: cara.wessels1991@gmail.com (C. Wessels), lindsay.penrose@ttuhsc.edu (L. Penrose), Khaliq.ahmad@ttuhsc.edu (K. Ahmad), samuel.prien@ttuhsc.edu (S. Prien).

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determine embryo viability in mice zygotes based on embryo buoyancy [5,6,7]. NEAT was designed from experimentation with a specific gravity system assembled in our laboratory (Fig. 1) [5]. The objective of the current study was to determine if NEAT can be used to detect embryo survival of cryopreservation in mice and ovine blastocysts, allowing a more objective determination of embryo viability after cryopreservation.

2. Materials and methods

Experimental protocols were approved by the Texas Tech University Animal Care and Use Committee.

2.1. Using NEAT to estimate embryo quality in mouse model

Zygote stage embryos (n = 169) were grown to blastocysts after being harvested from thirty, six-eight week old wild-type CB6F1 mice (Charles Rivers; Burlington, MA) stimulated using standard protocols. In brief, mice were allowed to acclimatize after their arrival before being hyper-stimulated with 5mlU FSH followed 24 h later by 5mlU of hCG to stimulate ovulation, and mice were mated to males of the same strain. 24 h after mating, females were euthanized and embryos were retrieved from the oviducts by micro dissection. Initial zygote buoyancy was determined by using NEAT, as previously described [5]. Zygotes were placed at the top of the NEAT chamber with a Stripper pipette (Origo; Malov, Denmark) with 140uL pipette tip (Cook; Bloomington, IN) by laying blastocyst in the center of the convex meniscus of Modified Ham's F-10 media (Irvine Scientific; Santa Anna, CA). Their decent through a fixed distance of 1 cm was timed with a stopwatch to determine their relative buoyancy. Time was determined visually through a dissection microscope (Nikon Instruments, Inc; Melville, NY) oriented horizontally towards the 1 cm timing zone on NEAT chamber with a boom arm stand. The zygotes were then cultured in 0.05 mL micro-droplets of Global Media (LifeGlobal; Guilford, CT) for a period of up to five days under conditions of 37 °C, with 95% relative humidity and 6% CO_2. Verification of development was checked daily. Zygotes developed into blastocysts and were used in further studies. NEAT was performed on all non-expanded blastocysts and buoyancy was determined based on descent time. The blastocysts were then dehydrated with Global Blastocyst Fast Freeze Kit (LifeGlobal; Guilford, CT) using standard protocols and cryopreserved in liquid nitrogen. Blastocysts were allowed a minimum of two weeks

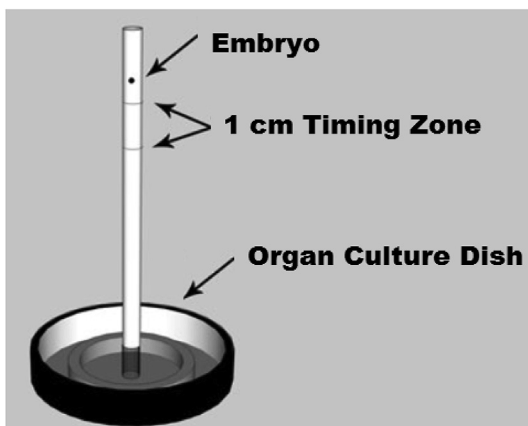

Fig. 1. Specific gravity system used in NEAT. Sealed device is filled from the top with embryo culture media. Embryo is placed at the top of the open end of the chamber and time as it descends through the timing zone. After measurements are taken, the lid can be removed and embryo can be found in the bottom of the central well of the organ culture dish.

in storage before thawing with Global Blastocysts Fast Freeze Thawing Kit (LifeGlobal; Guilford, CT) according to protocol. The buoyancy of the thawed blastocysts was determined by exposing them to the NEAT a third time, before returning them to pre-freeze culture conditions for an additional 24 h. At the end of the 24 h, blastocysts were assessed for survival. Blastocysts that degenerated in culture or failed to expand post-thaw were considered nonviable. Blastocysts that both expanded and hatched out of the zona pellucida post-thaw were assessed as viable and potentially capable of producing a pregnancy.

2.2. Preliminary trial using NEAT to estimate embryo quality in ovine pregnancy

In this preliminary ovine pregnancy study, all experiments were conducted during routine processing of embryos at a commercial ovine facility. In a previous breeding season, donor ewes were superovulated and laparoscopically inseminated in utero. Embryos were collected by surgically 6 days after insemination and frozen with Virgo Ethylene Glycol Freeze Plus with Sucrose (Bioniche; Pullman, Wa, USA). Embryos remained in liquid nitrogen storage until time of experimentation.

The specific gravity device necessary for NEAT was filled with 37 °C Virgo Holding Plus media (VHP-Bioniche; Pullman, Wa, USA). Fourteen frozen ovine blastocysts were thawed according to standard protocol. Thawed blastocysts were placed in VHP media in standard culture conditions, until ready for NEAT to be performed (wait time did not exceed 30 min). Thawed ovine blastocysts were dropped individually through the system. Individual blastocysts were retrieved in central well of organ culture dish following recording of their descent time. After all blastocysts (n = 14) were assessed using NEAT, blastocysts of similar descent times were paired and transferred into recipients as twins. All embryos were transferred to recipient ewes after laparoscopic examination and intra-abdominally inserted in the uterine horn. Pregnancy was determined by utilizing a DG^{29} blood test and multiple gestations were verified at lambing.

3. Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS ver. 12; Chicago, IL). The basic analysis was a two-way analysis of variance of treatment by time using a *P*-value of 0.05 for significance. In case of significance by the original analysis, the differences within time or treatment were reanalyzed with either Student's *t*-test or one-way analysis of variance with Tukey's means separation as appropriate.

4. Results

In the mouse model, 169 mice blastocysts were exposed to NEAT before freezing. There was no difference in pre-freeze drop times between viable and non-viable blastocyst, as determined by embryo hatching from zona pellucida (Fig. 2; $P = 0.10$). However, when blastocysts were re-exposed to NEAT after being frozen for a minimum of two weeks and thawed, those embryos which descended more slowly hatched from zona pellucida at a higher rate than the blastocysts with more rapid descent times (Fig. 2; $P < 0.001$). In a comparison, the percentage change in descent time from pre-freeze to post-thaw showed a significant decrease in buoyancy in non-viable blastocysts as compared to viable blastocysts. Blastocysts whose post-thaw descent time showed less change when compared to their pre-freeze time demonstrated viability by hatching from their zona pellucida at a greater rate than blastocysts who experienced a change from initial descent time.

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