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## Comparative Results of Survival of Vitrified Biopsied Goat Embryos and Mouse Morulae

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**Abstract:** The purpose of this study was to find an efficient cryoprotectant and a suitable procedure for vitrification of mouse morulae. The method found with mouse morulae was then applied to the cryopreservation of biopsied goat embryos. Mouse morulae were exposed, in 1- or 2-step, to the ethylene glycol (EG)-based solutions EFS30 or EDFS30, which contained 30% EG and 15% EG plus 15% dimethylsulphoxide (DMSO), respectively, in phosphate buffer saline (PBS) containing 30% (w/v) Ficoll and 0.5 M sucrose, cooled in liquid nitrogen and warmed up rapidly. After warming, the rate of the hatched blastocyst formation in mouse morulae vitrified with EFS30 (54.0%) by the 2-step method was significantly higher compared to those vitrified with EFS30 (30.4%) and EDFS30 (27.7%) by the 1-step method ( $P < 0.05$ ). According to those results, biopsied goat embryos were vitrified in EFS30 using the 2-step method. The results showed that the developmental blastocyst rate of the post-thawed embryos was 47.1%. It may be concluded that goat embryos were vitrified safely in an ethylene glycol-based solution (EFS30) by the 2-step method.

**Key Words:** Mouse morulae, biopsied goat embryos, vitrification, cryopreservation

### Introduction

Since the late 1970s, cryopreservation techniques have been established for embryos of most species at different developmental stages, and for more sensitive embryos such as in vitro produced, cloned, or biopsied embryos (1-6). In addition, frozen mouse morulae serve as useful models for the cryopreservation of livestock and human embryos (7). Successful cryopreservation of mouse morulae using EG or EG+DMSO as the permeating agent in the straw method was reported (7). However, for the survival of the vitrified and warmed embryos, vitrification procedure, such as the composition of vitrification solution and/or exposure time, should be improved.

In goats, the first cryopreservation of embryos was achieved by slow freezing in 1976. Later, Yuswiati and Holtz (8) and Traldi et al. (9) reported the successful freezing of goat embryos by vitrification technique. In 2001, the transfer of goat embryos using the open pulled straw (OPS) technique was performed by El-Gayar and Holtz (10). However, the survival rate after the transfer

of vitrified goat embryos was lower compared to those obtained by slow freezing (11,12). Reliable methods of cryopreservation of goat embryos, especially biopsied goat embryos, are still under development.

In the present study, using mouse embryos as a model, experiments were performed to find a convenient cryoprotectant and convenient conditions for exposing embryos to the vitrification solution. Based on the findings, biopsied goat embryos were subsequently vitrified. After thawing, viable embryos were transferred to the recipient goat.

### Materials and Methods

#### Preparation of mouse embryos

Except where otherwise indicated, all chemicals and culture media were obtained from Sigma Chemical Co. (St. Louis, MO, USA). ICR strain Kunming mice were purchased from Zhejiang Experimental Animal Center (Certificate No.22-2001001, Hangzhou, China). The mice were kept in light- and temperature-controlled

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conditions (14 h light: 10 h dark;  $23 \pm 2$  °C) and provided with food and water ad libitum. Sex mature females were given an intraperitoneal injection (10 IU) of pregnant mare serum gonadotrophin (PMSG) (Ningbo Hormone Products Co., Ltd., China). Forty-eight hours later, the mice were given intra peritoneal injection (10 IU) of human chorionic gonadotrophin (hCG) (Ningbo Hormone Products Co., Ltd., China). The females were mated with the males from the same strain and were inspected for the presence of the vaginal plug. Morulae were collected from the mated females 72 to 82 h after hCG injection. The embryos were flushed from the excised uterus with Dulbecco's phosphate buffer saline (PBS) (Gibco, Grand Island, New York, USA). Only morphologically normal embryos were selected.

#### Vitrification solution

As cryoprotectants, 2 permeating agents (DMSO and EG) were prepared. Vitrification solutions used were EFS30 and EDF30, which consisted of 30% (w/v) EG and 15% (w/v) EG plus 15% (w/v) DMSO, respectively, in a PBS solution containing 30% (w/v) Ficoll (average molecular weight 70,000) and 0.5 M sucrose. Moreover, 10% EG + 10% D, which contained 10% (v/v) EG and 10% (v/v) DMSO, in PBS containing 3 mg/ml bovine serum albumin (BSA) was used.

#### Vitrification of mouse embryos

Vitrification was performed according to the method developed by Zhou et al. (7) and Kasai et al. (13) for mouse embryos. The room temperature was adjusted in order to equilibrate the instruments and all solutions at  $25 \pm 0.5$  °C. For the 1-step method, embryos were vitrified consecutively in EFS30 and EDF30 for 40 s in plastic 0.25 ml straws. The average number of embryos loaded in each straw was about 3 to 5. The straws were sealed and immediately plunged directly into liquid nitrogen.

For the 2-step method, mouse morulae were first pre-equilibrated in 10% EG for 5 min and then morulae were exposed to EFS30 for 40 s, or first pre-equilibrated in 10% EG plus 10% D for 0.5 min and then exposed to EDF30 for 40 s.

For warming, straws were held 10 s in air, followed by 10 s in 37 °C water bath. The cryoprotectants were removed by placing the embryos in 0.5 M sucrose in PBS for 5 min. Embryos recovered after vitrification were washed and cultured in fresh medium under paraffin oil in a culture dish in an incubator at 37 °C in an

atmosphere of 5% CO<sub>2</sub> in air. Within 48 to 72 h, the embryos were assessed for their morphological appearance under a stereomicroscope. Then the survival of the embryos was assessed by their ability to develop to hatched blastocysts in culture.

#### Goat embryos

Synchronization of estrus in donor and recipient goats, Parous Boer goats aged 2-4 years, was induced by intravaginal progestagen treatment with a vaginal sponge containing 40 mg FGA (Chronogest<sup>®</sup>, Intervet, Angers, France) inserted for 12 days (day 0, sponge insertion). The donors were superovulated with a total dose of 320 mg FSH, consecutive injections of 100, 100, 50, 50, 10, and 10 mg at 12 h intervals.

Embryos were collected transcervically 6 to 8 days after induced estrus as described by Holtz et al. (14) and Suyadi et al. (15). This involved the induction of luteolysis with prostaglandin F<sub>2 $\alpha$</sub>  followed, 20 h later, by transcervical insertion of a flushing catheter and 10 flushes of each uterine horn. The embryos were classified according to the stage of development and morphological appearance 2 to 4 h after collection. Only morphologically normal embryos were selected for biopsy and vitrification (compact morulae).

One embryo at a time was picked out from the TCM199 medium and rinsed 5 times in M2 flushing medium devoid of BSA before being transferred to a drop of M2 medium on a level glass plate placed on the warming stage of an inverted microscope (Leica, Germany).

Approximately 6 to 15 embryo cells were removed from each embryo by a micro-scalpel (Feather Safety Razor Co., Ltd., Osaka, Japan). After removal from the manipulation medium, embryos were cultured in TCM199 for 5 h before the subsequent step of the experiment.

#### Vitrification of goat embryos

Embryos were treated at a room temperature of 25-27 °C for 5 min in PBS. From the results of the experiments on mouse embryos, an ethylene glycol-based solution, EFS30, was selected as the vitrification solution for goat embryos. The goat embryos were vitrified in the 2-step method at room temperature as follows: 10% EG for 5 min and EFS30 for 40 s. The straws were sealed and positioned horizontally in vapour phase above the liquid nitrogen for 30 s before being plunged into liquid nitrogen.

Embryos were thawed and recovered as described above for mouse embryos, except that the straws were kept in air for 10 s before immersion in water at 37 °C for 10 s. For manipulating embryos, sterile culture dishes were used instead of watch glasses. The embryo quality was evaluated under a stereomicroscope according to morphological criteria and all degenerated embryos, i.e. those with abnormal morphology and disrupted membrane were eliminated. Only morphologically normal embryos were transferred to recipient goats.

#### Transfer of goat embryos

For each recipient, 1 embryo was aspirated with 20 µl PBS in a glass capillary connected to a 1 ml syringe and introduced surgically into the tip of the uterine horn ipsilateral to the ovary bearing at least 1 functional corpus luteum. Pregnancy was diagnosed echographically on day 40 of gestation using a real time ultrasonography (Aloka SSD 500 with 7.5 MHz linear-array transducer) as described by Medan et al. (16).

#### Statistical analysis

Results were analyzed by chi-square test unless the expected frequency was less than 5, in which case Fisher's

exact probability test was used. The probability value of  $P < 0.05$  was taken as statistically significant.

## Results

#### Vitrification of mouse embryos

As shown in Table 1, EFS30 and EDF30 were used to vitrify mouse morulae by the 1-step or 2-step method. The proportion of the hatched blastocysts after vitrification with EFS30 (30.4%, 14/46) and EDF30 (27.7%, 13/47) by the 1-step method was significantly lower compared to the 2-step method (54.0%, 27/50) ( $P < 0.05$ ). The survival rate of the embryos developed to hatched blastocyst vitrified with EFS30 (54.0%) was higher compared to those vitrified with EDF30 (40.8%, 20/49) by the 2-step method, but the difference was not statistically significant. In light of these results, we employed EFS30 and the 2-step method for the subsequent vitrification experiment.

#### Vitrification of goat embryos

The survival of the biopsied goat embryos after vitrification with EFS30 is shown in Table 2. In total, 17 biopsied embryos were vitrified and 8 of them were

Table 1. In vitro development of mouse morulae by vitrification.

Vitrification solutions	Equilibration time(s)	Number of embryos	Without zona (%)	Developmental date <sup>3</sup> (%)
EFS30				
1-step	40	46	1(2)	14(30.4) <sup>a</sup>
2-step <sup>1</sup>	40	50	0(0)	27(54.0) <sup>bc</sup>
EDFS30				
1-step	40	47	0(0)	13(27.7) <sup>a</sup>
2-step <sup>2</sup>	40	49	0(0)	20(40.8) <sup>ac</sup>

<sup>1</sup> The mouse morulae were vitrified after the first pretreatment with 10% EG for 5 min, then exposed to EFS30.

<sup>2</sup> The mouse morulae were vitrified after the first pretreatment with 10% EG + 10% DMSO for 0.5 min, then exposed to EDF30.

<sup>3</sup> Developmental rates to hatched blastocysts

<sup>a,b,c</sup> Values with different superscripts in the same column are significantly different ( $P < 0.05$ ).

Table 2. Pregnancy rate of and embryo survival rate of goat embryos by vitrification.

Embryos	Number of vitrified	Developmental blastocysts rate (%)	Pregnancy rate (%)	Embryo survival rate <sup>1</sup> (%)
Biopsied	17	8(47.1)	3(37.5)	2(25.0) <sup>a</sup>
Fresh intact	18	–	11(61.1)	8(44.4) <sup>a</sup>

<sup>1</sup> Embryo survival rate = The number of kids born/transferred recipient ratio.

<sup>a</sup> Values with different superscripts in the same column are significantly different.

developmental to blastocysts, suggesting that the moderate cooling methods adopted and in vitro culture after biopsy may have been effective in preventing fracture damage. After transfer, pregnancy rate of the biopsied embryos and fresh intact embryos was 37.5% (3/8) and 61.1% (11/18), respectively. No statistically significant difference was observed between the embryo survival rates of the vitrified biopsied embryos (25.0%, 2/8) and fresh intact embryos (44.4%, 8/18).

## Discussion

The survival rate of vitrified embryos depends on several mechanisms of cell injury, such as the chemical toxicity of the cryoprotectant, intracellular ice formation, fracture damage, and osmotic swelling during the removal of the cryoprotectant. In order to find suitable conditions for vitrification of mouse and biopsied goat embryos, we examined the effects of the vitrification solution on the post-warming survival of vitrified mouse morulae by the 1-step or 2-step method, and find a suitable procedure for vitrification of biopsied goat embryos.

The vitrification solutions used in our experiments contained a mixture of permeating and nonpermeating cryoprotectants. Permeating agents, such as EG and DMSO are organic solutes responsible for protecting the intracellular organelles of the cells during cooling and warming prior to and after storage in LN<sub>2</sub> (17). Previous studies have found enhanced permeability to EG compared to propylene glycol, glycerol, and DMSO (18, 19). In the present study, mouse morulae were vitrified in EFS30 or EDF30 by the 1-step or 2-step method at room temperature. As shown in Table 1, EFS30 solutions gave better protection against mouse morulae during vitrification by the 2-step method compared to EDF30 by the 1-step or 2-step method at room temperature. It confirmed the low toxicity of EG for mouse morulae, and that EFS30 solutions are suitable to vitrify embryos. This agreed with a previous study where ethylene glycol was superior to glycerol and DMSO as a cryoprotectant for the freezing of embryos (20).

According to Cseh et al (21), the most suitable developmental stage for vitrification was the compacted morula. Therefore, we used mouse morulae as a model to screen feasible vitrified methods for biopsied goat embryos. Several studies have demonstrated that early

stage embryos (2- to 4-cell stage), morulae, and blastocysts can be vitrified (9,10,22-24). To our knowledge, however, there is no report of successful cryopreservation of biopsied goat embryos.

In ovine, 5 to 8 h of in vitro culturing after cell sampling prior to cryopreservation improved the cryoviability of the biopsied embryos (25). The cell biopsy did not compromise embryo viability and subsequent vitrification, particularly when embryos were at the blastocyst stage. It is also reported that, in bovine, 1.25 to 10 h (especially 2.5 to 5 h) of culture after biopsy improved the tolerance of the biopsied embryos to the vitrification treatment (26). In this study, after the biopsy of goat embryos, we cultured embryos for 5 h. Since the membrane and structure of the biopsied embryo cells damaged by surgery may recover during these culture periods, the tolerance of the embryos may be improved. Biopsied goat embryo development to blastocyst rate after vitrification observed in the present study (47.1%) was comparable to the results reported previously by Zhu et al. (27) (51.8%) with EFS40 in ovine.

In our embryo transfer program, fresh intact embryos were first assigned for immediate transfer and the rest of the embryos were subjected to biopsy and vitrification. After transfer, pregnancy rates of the biopsied goat embryos obtained in our study (35.0%) were slightly lower than those reported by Traldi et al. (28) for vitrified intact goat embryos (56%). The embryo survival rate in our study was 25.0% in the biopsied goat embryos that were exposed to a 10% EG solution for 5 min followed by EFS30 for 40 s and it is 44.4% in the fresh intact embryos, which is not a statistically significant difference. This demonstrated that membrane and structure of the biopsied embryo cells damaged by surgery may recover after in vitro culture. However, the embryo survival rate of thawed embryos in the present study was lower compared to those reported by Guignot et al. (24) with standard vitrification (48%) and vitrification with the addition of 0.4 M sucrose in the last step (60%). In conclusion, these differences may result from the small number of embryos, different species of recipients, and the transfer techniques.

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