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Cryopreservation of Two Cells Stage Mouse Embryos: An Experience in Center of Molecular Immunology, Cuba

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Abstract

Over the past three decades, the generation of genetically modified tumor models has increased. Embryo cryopreservation is essential technique to save and handle the increasing the mice with spontaneous or induced mutations. This technology serves to reduce maintenance costs and the mouse colony is protected from infections. The Center of Molecular Immunology (CIM, Havana, Cuba) have developed several immunotherapeutic projects with focus in oncology studies in some special biomodels. However, there is no experience in the field of cryopreservation methods as a tool to increase the quality to work with genetically engineered mouse models. The objective of this work is to establish the technique of cryopreservation of murine embryos at the CIM. The maintenance and handling of animals from the National Laboratory Animal Production Center (CENPALAB), was carried under the regulations of the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the CIM. We used a vitrification method for mouse embryos in liquid nitrogen with etylenglycol medium. In addition, a Ficoll and sucrose was employed to prevent the devitrification process. The freeze embryos since four months were transferred to the pseudopregnant (NMRI/Cenp mice). The efficiency of the defrosting process was greater than 80%. As a result of the embryo transfer, the healthy born pups were obtained. These results suggest that the cryopreservation technique was established at the CIM and it will promote high standards of biomedical research.

Keywords: Cryopreservation; Embryos; Mouse; Biomodels

Introduction

The cryopreservation of murine embryos is an important tool for high standards of animal experimentation. This technique supports the work with the mouse obtained by genetic modifications. Cryopreservation methods allow the conservation of a large number of strains of mice reducing their maintenance costs and protecting them from infections [1]. This procedure is based on the maintenance of viability and cellular functionality at low temperatures [1].

The first reports of mouse embryos cryopreservation was in the 70s [2]. A slow freezing and vitrification are the two methods to preserve the cells at low temperatures for an indefinite time [2,3]. Particularly, the vitrification is a quick freezing alternative, which it has shown a better embryo survival than that obtained with the slow freezing [4,5]. The advantage of vitrification process refers to the physical event of solidifying a solution at low temperatures without the formation of ice crystals [4,6]. The phenomenon can be considered as an extreme increase in viscosity, which requires high rates of cooling and heating [6]. This method is fast, simple and offers an economic advantage since it does not require special equipment [4]. However, this is not a problem-free assay since small variations in any of the phases of the process can affect the viability of the embryos and thus the non-acquisition of births due to their transfer [4]. This Technique requires extensive practical skills. The success of vitrification is also operator-dependent, and varies from laboratory to laboratory.

During many years, the vitrification technique has been modified. In 1990, to minimize the potential toxicity of vitrification solutions, the focus was to the combined use of mixed permeating CPAs (e.g., ethylene glycol [EG], dimethyl sulfoxide [DMSO] and glycerol), as well as non-permeating solutes (e.g., sucrose and ficoll) that factored into reducing toxicity potential of individual vitrification solutions [6]. Mochida [7] efforts to develop an effective procedure working under room temperature conditions and cryotubes.

The Center for Molecular Immunology (CIM) require increasing the use of biomodels obtained by techniques of genetic engineering and assisted reproduction. However, there is no experience in the field of cryopreservation methods as a tool to minimize the high cost of maintaining the special biomodels. The objective of this study was to standardize the embryo cryopreservation technique at the animal facility in CIM.

Materials and Methods

Animals

The females and males were 8-9 weeks old. NMRI/Cenp, BALB/c/Cenp and C57BL/6/Cenp were obtained from CENPALAB (Cuba). The maintenance and handling were carried out according to the regulations established by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) at the CIM. The manipulation of animals was done considering the circadian rhythms of 10 hours of light and 14 hours of darkness. They were given free access to feed and drinking water. The clinical evaluation was done every day.

Collection of two-cell stage embryos

Prepare 2-cell mouse embryos by natural mating. The mucous plug was detected by periodic check-ups. The females with mucous plug were isolated for 24 hours and then, they were sacrificed. The ovaries and uterus were removed using a stereomicroscope (Olympus SZ61). The oviduct was perfused with M2 medium (SIGMA-ALDRICH) and the

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embryos were removed. Recovered embryos were classified based on the morphological criteria as Ghanem reported [8].

Embryo freezing (Vitrification)

The procedures for cryopreservation of two cells stage mouse embryos by Ethylene glycol-based vitrification was performed in accordance with the method described by Mochida et al. [7]. All solution was prepared according to Mochida et al. [7]. All reagents used in the preparation of solutions were purchased from Sigma Chemical Co. (St Louis, MO).

The embryos to be frozen were exposed to Balance solution Ethylene glycol-Ficoll-Sucrose 20 (EFS20), 2 minutes (min). Then, the embryos were checked morphologically by a stereomicroscope, they should be slightly shrunken [7]. Later, the embryos were placed to cryotubes (Sumitomo Bakelite Co. Ltd., contained Ethylene glycol-Ficoll-Sucrose 40 vitrification solution (EFS40), 2 min. They were cooled at 0°C for 1 min. Next, the cryotubes were placed directly into liquid nitrogen (LN_2). The vitrified embryos were stored in liquid nitrogen for at least four months.

Defrosting

The vitrified embryos were warmed by shifting the cryotubes from liquid nitrogen to room temperature (25°C ± 0.5°C). Previously, the KSOM medium (EmbryoMax® KSOM Mouse Embryo Media, Chemicon/Millipore) was coated with liquid paraffin and incubated at 37°C, 5% CO_2. TS1 solution (heated at 37°C) was added to the cryogenic tube containing embryos, preserved in LN_2. Next, the contents of the cryogenic tube were transferred to a Petri dish for 3 min. Recovered embryos were classified based on the morphological criteria as the guidelines in the Manual International Embryo Transfer Society [9]. The embryos were washed (TS1 and TS2), 3 min/each. Next, they were transferred to KSOM and incubated at 37°C, 5% CO_2, 10 min. The *in vivo* development of embryos that survived after cryopreservation was examined. The embryos continued in an incubator until their transfer.

Embryo transfer

Pseudopregnant recipients with vaginal plug were selected for embryo transfer. The rederivation was performed by the two cells stage murine embryos transfers. The recipient (NMRI mice) were anesthetized with cocktail. For a 2 ml vial using ketamine (50 mg/ml, 1 ml)-diazepam (10 mg/2 ml, 0.8 ml)-atropine (0.5 mg/ml, 0.2 ml) (AICA Laboratories). Each animal received an intraperitonal injection of cocktail (0.05 ml). The skin incision (1 cm) was made parallel to the dorsal midline for exposing oviduct. The oviduct wall was punctured and the loaded pipette was inserted into the hole. The microcapillary glass containing embryos (8 embryos/oviduct) was discharged into the oviduct until air bubbles (3) became visible in the ampullae. The oviduct, ovary and fat were introduced into their cavity and the same procedure was performed for the other ovary. The wound was sutured with Vetacryl tissue adhesive (Biomaterials Center of the Havana University). Daily, the pregnancy was checked after embryo transfer by technician. The survival rates and development of two cell stage embryos are expressed as percentage.

Results

NMRI/Cenp, C57Bl/6/Cenp and BALB/c/Cenp lines were selected to establishment this cryopreservation technique. The technique of vitrification is done in liquid nitrogen in a medium of Ethylene Glycol and Ficoll. The total of donor females and the number of embryos collected by the murine lines were different. However, in all cases, the total of frozen embryos per murine line was more than 90% of collected embryos. Table 1 summarizes the efficacy of vitrification method used at the CIM. Figure 1 indicates the dehydrated two cell stage embryos (NMRI) presenting a shrunken morphology.

Strain	No. of donors (females)	No. of embryos collected	No. of embryos vitrified	Efficiency
				Total (%)
NMRI/Cenp	22	325	300	92,3
C57BL/6/ Cenp	5	34	31	91,2
BALB/c/Cenp	24	177	162	91,5
No: Number; (%): Percentage				

Table 1: Vitrification of embryos from common murine strains.

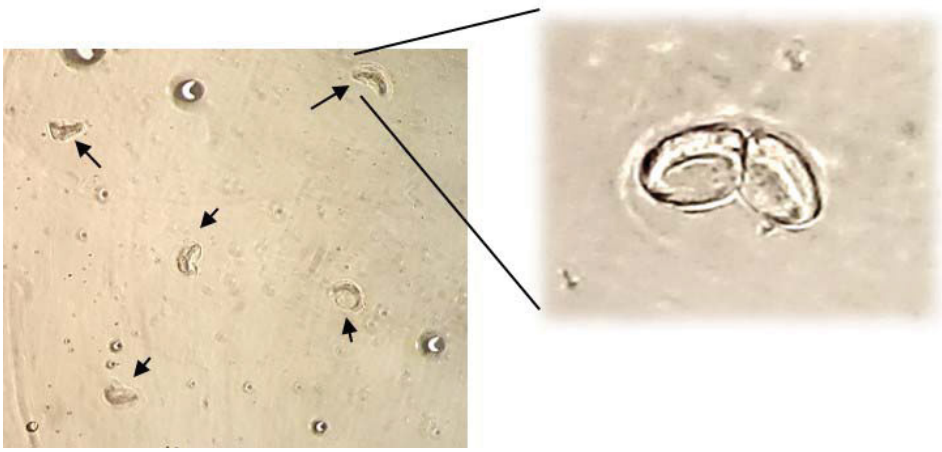

Figure 1: Morphology of mouse embryos in vitrification process. Two-cell stage mouse embryos from NMRI/Cenp strain in vitrification medium (EFS20). The method was performed at room temperature. The black arrows indicate dehydrated embryos with a shrunken morphology. Black bar=200x.

Subsequently, in order to check the viability of the vitrification methods, four-month embryos vitrified from the NMRI and C57BL/6 lines were thawed. Table 2 shows that the viability percentage (efficiency) of the embryos vitrified by lines. The recovery process of frozen embryos was greater than 80%. NMRI (84%) and C57BL/6 (87%). To corroborate the viability of two cell stage murine embryos, the blastocyts were cultured in KSOM medium during the 18 hours. The two cell stage embryos passed to the next stage (four-cell embryos) (Figure 2). In this case, the viability of the thawed embryos was greater than 85%: NMRI (89.7%) and C57BL/6 (96.3%) (Table 2).

The final step of the embryo freezing technique is the transfer process. All live embryos vitrified-thawed were transferred to pseudo-pregnant recipient NMRI females two days after. As the results, we obtained live offspring and NMRI (6%) and C57BL/6 (19%) respectively (Table 3). In addition, the pups had a good health and it never presented symptoms of physical degeneration. Figure 3 illustrates the results the transfer process the C57BL/6 embryos to NMRI females.

Discussion

The special biomodels are the second most common type of mouse models in oncology research [10]. Several vaccine and monoclonal antibodies have been developed at the CIM [11-14]. Their preclinical evaluation continues with focus in special biomodels. The high maintenance of transgenic mice is an important issue associated with the preclinical research. In the last years, the cryopreservation of mouse embryos is a significant tool to work with strain mice obtained by genetic modifications. This work shows a vitrification method for mouse embryos cryopreservation stablishmed in the Animal House at the CIM.

Vitrification is a method extremely rapid, in which the embryos are included in a highly concentrated solution of cryoprotectants, solidifying during cooling without the formation of ice crystals [3,4]. This method does not require any expensive/special equipment. Although, it has main two limitations: Both the duration of mouse embryos exposure to the cryoprotective solutions and the temperature of the cryoprotective solutions must be precisely controlled to minimize potentially toxic effects. Second, it requires highly skilled technicians for processing, handling, and storage.

Since the first report of mouse embryos in 70s [2]; several technical improvements have been made to increase the survivability of embryos after thawing. One of the most successful modifications was achieved by use of ethylene glycol as a cryoprotectan because of its low toxicity [15]. The first ethylene glycol-based vitrification was developed by Kasai [16]. This original method was optimized for plastic straws as container, however Mochida et al. modified this method for cryotubes, which are more easily accessible and the benefits of high embryos survival after vitrifying and thawing (or liquefying, more precisely) at the ambient temperature [7]. Several authors have been reported that the survival of mouse embryos preserved by freezing or vitrification ranges between 16% and 54% [3]. In contrast, Mochida [7] reported a high survivability of embryos after thawing and its broad applicability to different strains of mice: C57BL/6J (87%), BALB/c (84%) and ICR (90%).

Strain	No. of vitrified embryos/cryotube	No. of thawing embryos Total (%)	No. of live embryos Total (%)
NMRI/Cenp	150/5	126 (84)	113 (89,7)
C57BL/6/Cenp	31/2	27 (87)	26 (96,3)
No: Number; (%): Percentage			

Table 2: Defrosting of vitrified embryos from common murine strains.

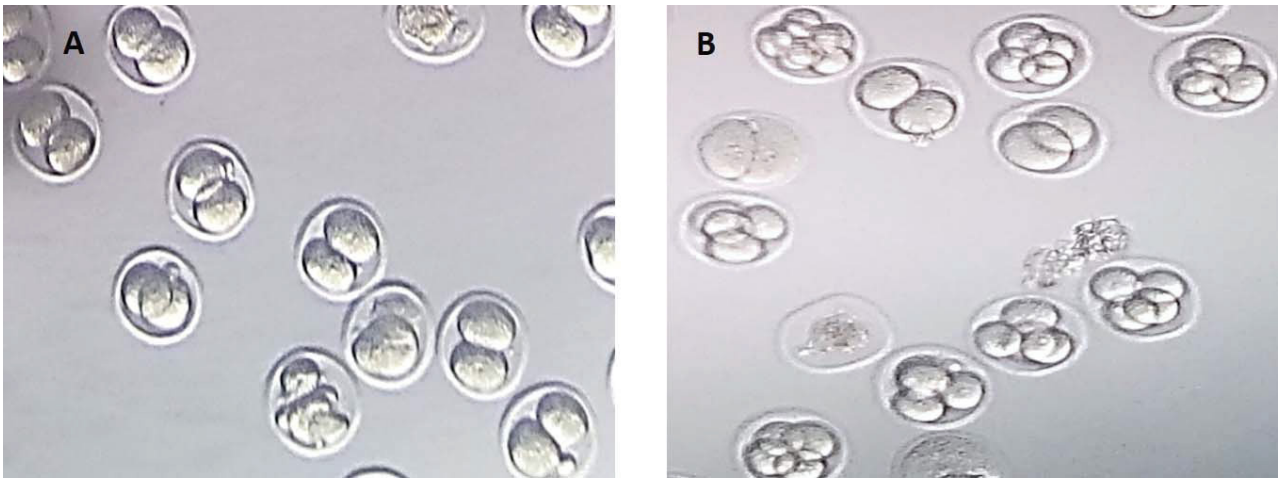

Figure 2: Morphology of embryos culture. Two-cell stage mouse embryos vitrified from NMRI/Cenp strain were thawing after four months. The embryos were culture in KSOM medium (37°C). (A) 10 min. (B) 18 h. The black arrows indicate the two-cell (A) and four-cell (B) embryos. Black bars=200x.

Strain	Transferred no. (%)	Live offspring No. (%)
NMRI/Cenp	109(96,46)	7(6,42)
C57BL/6/Cenp	26 (96)	5(19,23)
No: Number; (%): Percentage		

Table 3: Transfer of vitrified embryos from common murine strains.

Figure 3: Development of full-term offspring. Two-cell stage mouse embryos vitrified from C57BL/6/Cenp mice were transferred to NMRI/Cenp recipients. After fourteen days, these offspring had grown healthy.

In the Animal House at the CIM, there is not experience in cryopreservation of murine embryos, so we decided to establish the vitrification technique using the method described by Mochida [7]. To standardize the cryopreservation techniques we selected the common strain (NMRI, C57BL/6 and BALB/c). First, the efficacy of extraction, collection, and the embryos handling and dehydration process was more than 90%. Then, the efficiency of *in vitro*- and *in vivo*-development of murine embryos after thawing was more than 84% in two lines: NMRI and C57BL/6. This result are agree with others authors [1,17]. However, strain/genetic background and technical (protocol, media quality, and technician skill) are factors that could affect the efficiency of embryos cryopreservation [18]. In our experimental conditions, numerous gametes lost their viability in NMRI line, so future attempts might rely to increase the efficiency of this process by strain and extend it to other lines of mouse.

In regarding to the embryos transfer, Nagy [18] showed that the embryo transfers had a significantly lower pregnancy rate and implantation rate (4 and 1%, respectively). On the other hand, Mochida [7] reported that the transfer of embryos vitrified in C57BL/6J show 51.7% of live offspring. More recently, Sarvari et al. [19] described that the average rate of developing live newborns for mice was of 42%. For the first time, we obtained pups from two cell stage murine embryos vitrified at the CIM, although the efficiency of the transfer process was from six to 19% of live births. Previously, the direct transfer of two cells stage murine embryos was practiced by our technician team (data no show); however this result is low (quite) as compared to others laboratories. Several authors reported that this process may affected by a combination of biological and technical factors. Taking account this idea, the future studies will be aimed in two ways: first, to learn more about our strain and the second, to increase the level of experience and technical skill.

Conclusion

In summary, the offspring of the NMRI/Cenp and C57BL/6/Cenp strain obtained by the transfer of two cells stage murine embryos vitrified indicates that the technique was established in the CIM. Conclusions, the operation of this technique will allow us to work with a greater number of biomodels to increase the quality of animal experimentation.

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