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Original Article

Optimal freezing and thawing for the survival of peripheral nerves in severed rabbit limbs

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Abstract: This study aimed to investigate the optimal freezing and thawing procedures for the survival of peripheral nerves in severed rabbit limbs. Twenty New Zealand White rabbits were randomized into four groups: normal control, slow-freezing fast-thawing, slow-freezing slow-thawing, fast-freezing fast-thawing, with five animals in each group. The hind limbs of the rabbits were severed at 1 cm above the knee joint. The severed limbs were cryopreserved with various freezing and thawing procedures. The sciatic nerves were harvested and trypsinized into single nerve fibers for morphological evaluation. The cell viability of the nerve fibers was examined by staining with Calcein-AM and propidium iodide. The fluorescent intensity of the nerve fibers was measured with a laser scanning confocal microscope. The morphology of the nerve fibers in the slow-freezing fast-thawing group was very similar with that of the normal control group, with only mild demyelination. The slow-freezing fast-thawing group and slow-freezing slow-thawing group showed severely damaged nerve fibers. The fluorescent intensities of the nerve fibers was significantly different among the four groups, with a decreasing order of normal control, slow-freezing fast-thawing, slow-freezing slow-thawing, and fast-freezing fast-thawing ($P < 0.05$). Of the various cryopreservative procedures, slow-freezing fast thawing has the minimal effects on the survival of nerve fibers in severed rabbit limbs.

Keywords: Cryopreservation, nerve fibers, rabbit limbs, survival, fluorescent intensity

Introduction

In the recent decades, the booming economy in China has promoted a rapid increase of road traffic, and therefore, the incidence of car accident and trauma are also growing accordingly. Increasingly more victims of car accident suffer from severed limbs, fingers, or toes. Immediate replantation of severed limbs has been a common procedure with high success rate. However, replantation is a complex procedure and can last several hours or even longer, which is intolerable for those severely injured patients. These patients may need delayed replantation of the severed limbs when their general condition permits the very time-consuming procedure. Therefore, cryopreservation of severed limbs, fingers, and toes is important for the success of delayed replantation. However, successfully cryopreserved limbs and replantation has rarely been reported.

Previous studies have shown that cryopreservation at ultra-low temperatures can reserve the viability of cells and tissues [1]. The mechanism of cryopreservation is that low temperature can significantly slow down or even stop the enzymatic reactions in cells, leading to decreased cell metabolism [2]. At the temperature of liquid nitrogen of -196°C, all the intracellular biochemical reactions are ceased, and the cell metabolism is stopped. The cryopreserved cells at this temperature will not have neither apoptosis nor necrosis, and maintain their intact biological structures. However, the freezing and thawing processes can damage the cryopreserved cells and tissues.

During slow freezing, water nucleation occurs first in the extracellular fluid, which then decreases the volume of extracellular fluid. The decreased extracellular fluid disrupts the osmolarity balance between the intracellular and extracellular environments. The intracellular water then transfers to the extracellular environment, increasing the intracellular osmolarity and damaging the organelles. In the contrary, fast freezing induces water nucleation simultaneously in both the intracellular and extracellu-

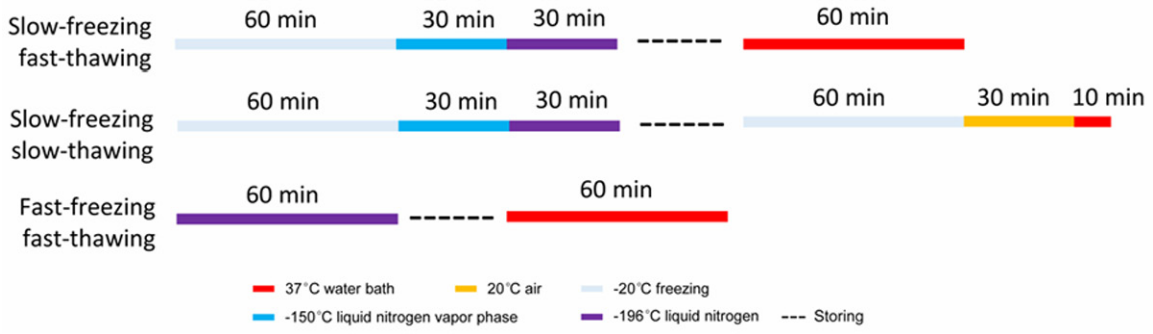

Figure 1. The three different freezing and thawing procedures used for the treatment of rabbit limbs.

lar environments. The formation of intracellular ice can damage the organelles cause cell injury. To prevent cryopreservation-induced cell damage, various cryoprotectants, such as glycerol and dimethyl sulfoxide (DMSO) have been applied. And specific freezing and thawing procedures have been established for different cell types and tissues.

Since the first successful cryopreservation of bovine sperm cells in 1949 [3], cryopreservation of single cells has been developed into a mature technology. In addition, cryopreservation of single tissues also undergoes rapid development. The cryopreservation of combined tissues is becoming possible on the basis of cell and tissue cryopreservation. The combined tissues may include skin, fat, tendon, muscle, bone, nerve, and vessel. The various cell and tissues types have different rate of heat conduction, which may cause damages to the tissues. The complex composition of combined tissues may pose an important challenge to the successful cryopreservation of severed limbs.

The survival of peripheral nerves is critical for the reestablishment of motor and sensory functions after limb replantation. The impact of freezing and thawing on the nerve fiber viability of cryopreserved limbs has not been investigated. We for the first time investigated the cryopreservation of combined tissues and the optimal freezing and thawing procedure for the survival of nerve fibers in severed rabbit limbs.

Materials and methods

Animals

Twenty New Zealand White rabbits weighed 2.5 to 3.0 kg. All the procedures were in compliance with the Guidelines for the capture, handling, and care of mammals as approved by The American Society of Mammalogists [4]. The experiment was approved by the ethics committee of our institution.

The rabbits were randomly assigned into four groups: normal control, slow-freezing fast-thawing, slow-freezing slow-thawing, fast-freezing fast-thawing, with five animals in each group. The rabbits were sacrificed by air embolism via the ear vein. The hind limbs were prepared with iodophor and then severed at 1 cm above the knee joint. Caution was used not to over-stretch the sciatic nerve.

Freezing and thawing

The severed limbs of the normal control group were not treated. For other three groups, the severed limbs were first perfused with heparin saline (25 U/ml) of 37°C and a pressure of 120 mmHg for 5 min via the femoral artery, and then perfused with cryoprotectant (RPMI-1640 medium supplemented with 10% DMSO [Sigma, USA] and 10% bovine serum) for 15 min of the same temperature and pressure. After perfusion, each limb was sealed in a plastic bag full of cryoprotectant and underwent different freezing and thawing procedures (**Figure 1**). After thawing, the limbs were perfused again with 5% glucose solution supplemented with 20% bovine serum of 37°C and 120 mmHg for 10 min.

Morphology of the nerve fibers

The sciatic nerve of 5 cm was harvested from each limb. The branches of the sciatic nerve were dissected and the epineurium was carefully removed. Then the sciatic nerve was incubated with 0.125% trypsin at 37°C for 15 min

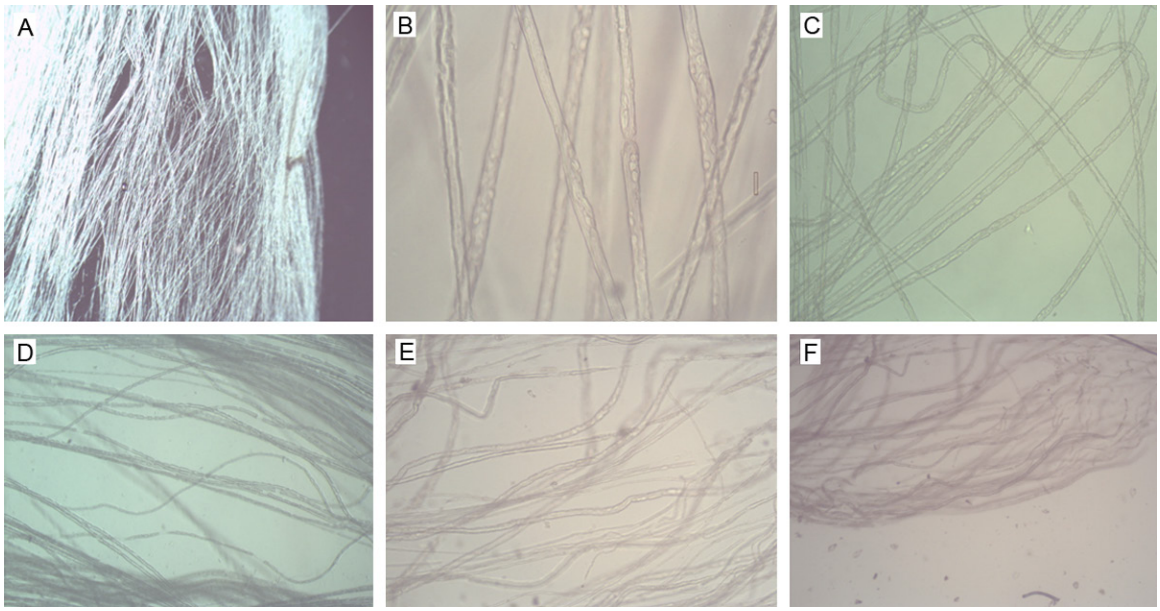

Figure 2. Morphology of the nerve fibers. A-C. The normal control group. D. The slow-freezing fast-thawing group. E. The slow-freezing slow-thawing group. F. The fast-freezing fast-thawing group.

to prepare the single nerve fibers. The digestion was stopped with adding 10% bovine serum for 10 min. The single nerve fibers were observed under a light microscope.

Calcein-AM and propidium iodide (PI) staining

It investigate the survival of the sciatic nerves, the nerve fibers were stained with calcein-AM (2 μmol/l) and PI 0028 (4 μmol/l) at 37°C for 15 min in dark. Then the nerve fibers were washed with phosphate buffer saline to remove the staining solution. The fluorescent intensity was measured under a laser scanning confocal microscope and analyzed with an image system (LAS-AF-Lite 2.6, Leica, Germany).

Statistical analysis

All data were expressed in mean ± standard deviation. Statistical analysis was performed with SPSS 17.0 software (SPSS, IL, US). Comparisons among the four groups were made using one-way ANOVA followed by LSD test. A $P < 0.05$ was considered statistically significant.

Results

Morphology of the nerve fibers

The nerve fibers of the control group showed smooth surfaces and intact myelin sheath. No exposed axons were found in the control group (**Figure 2A-C**). The slow-freezing fast-thawing group showed twisted nerve fibers with mile demyelination with occasional axon exposure or discontinuity (**Figure 2D**). In the slow-freezing slow-thawing group, severe demyelination and axon exposure and discontinuity were seen (**Figure 2E**). The morphology of nerve fibers in the fast-freezing fast-thawing group was similar with that of the slow-freezing slow-thawing group, with additional abundant cell debris (**Figure 2F**).

Vitality of the nerve fibers

The nerve fibers stained with calcein-AM and PI are shown in **Figure 3**. The fluorescent intensities of calcein-AM staining were measured and compared among the four groups, showing an decreasing order of normal control, slow-freezing fast-thawing, slow-freezing slow-thawing, and fast-freezing fast-thawing ($P < 0.05$; **Figure 4**).

Discussion

The first cryopreservation of embryos was reported in 1972 [5]. Since then, significant progresses have been made in the cryopreservation of various tissues [6-10]. Many types of tissues have been successfully cryopreserved, including ovary, sperm, embryo, skin, vessel,

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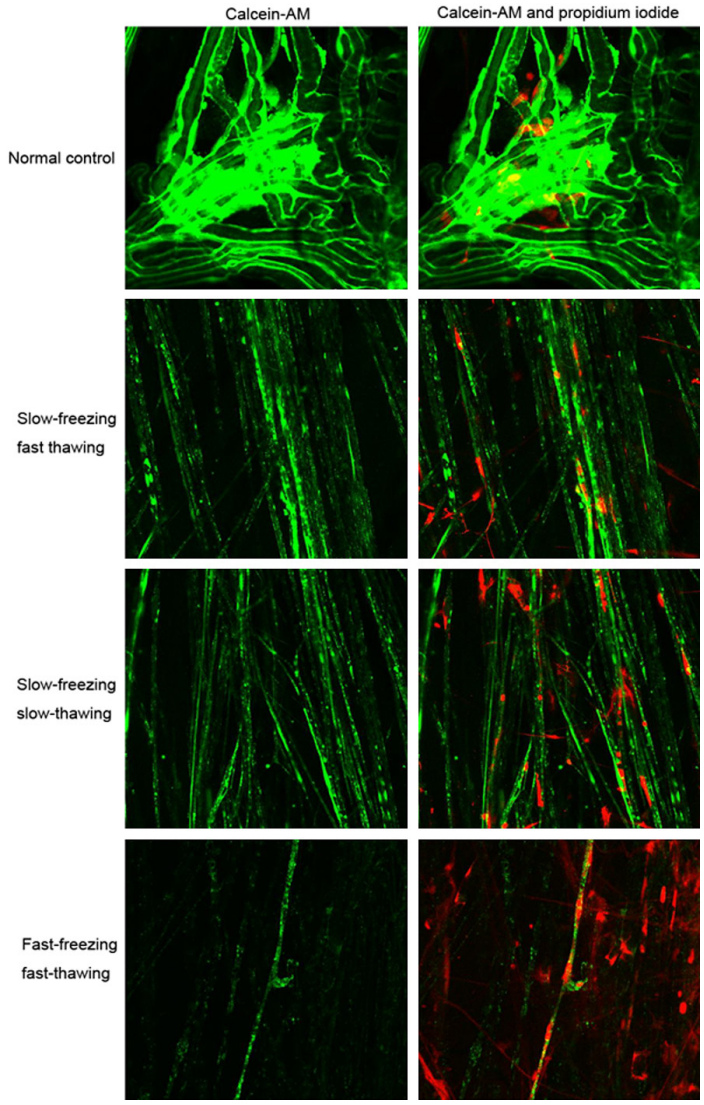

Figure 3. Calcein-AM and PI staining of the nerve fibers. Calcein-AM staining is the green fluorescence showing the vital nerve fibers. PI staining is the red fluorescence showing dead nerve fibers. The slow-freezing fast-thawing group had the best vitality and the least cell dead compared with the other cryopreservation groups.

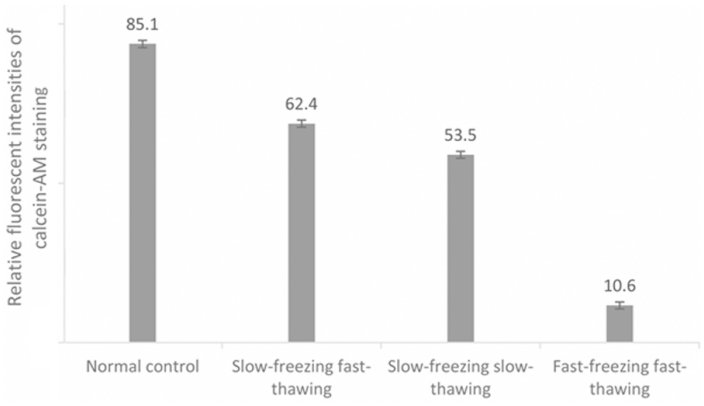

Figure 4. Relative fluorescent intensities of calcein-AM staining was in decreasing order of normal control, slow-freezing fast-thawing, slow-freezing slow-thawing, and fast-freezing fast-thawing ($P < 0.05$).

bone, cartilage, and tendon. With the development in cryoprotectants, the cryopreservation of a single tissue type has become a reliable and mature technology.

Cryopreservation of combined tissues can enable the establishment of tissue banks of organs and limbs. The tissue banks will become a rich source for transplantation materials. In addition, the severed limbs, fingers, and toes during various traumas can be cryopreserved for delayed replantation when the patients can tolerate the procedure. However, the cryopreservation of combined tissues has been a great challenge. Each tissue type has its own specific cryopreservation parameters, such as freezing and thawing rate, and type and concentration of the cryoprotectants. The inconsistent conduction rate of heat between different tissue types may results temperature variation and consequently cell damage. We have designed this study to investigate the effects of various freezing and thawing procedures on the survival of sciatic nerves in the severed rabbit limbs.

The viability of nervous tissues is critical for the restoration of motor and sensory functions after limb replantation. The regeneration of peripheral nerves requires intact axon conduits and vital Schwann cells. It has been shown that Schwann cells can secret various cytokines to promote the regeneration of axons [11]. In this study, we found that the freezing and thawing procedures disrupted the interiority of Schwann cells and myelin sheath, leading to the exposure of axons.

The slow-freeing fast-thawing procedure showed the minimal effects on the morphology and vitality of the nerve fibers.

We used calcein-AM and PI staining to demonstrate the vital and dead cells, respectively. Calcein-AM is the acetomethoxy derivate of calcein and can be transported through the cellular membrane into live cells, which is useful for testing of cell viability. Intracellular esterases remove the acetomethoxy group, then calcein-AM gets trapped inside and gives out strong green fluorescence. As dead cells lack active esterases, only live cells are labeled by calcein-AM [12]. PI is membrane impermeant and generally excluded from viable cells. PI can bind to nucleic acids and emit red fluorescence [13].

In conclusion, our study showed at among the three cryopreservation procedures, slow-freezing fast-thawing best reserved the structures of the nerve fibers and its viability in severed rabbit limbs. Further investigation is needed to find out the effects of cryopreservation on the survival of other tissues in the limbs.

Disclosure of conflict of interest

None.

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