Application of Cryopreservation Technique in the Preservation of Rat Limbs

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Research

Keywords: Cryopreservation, University of Wisconsin, Limbs, Replantation

Posted Date: August 25th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-63528/v1

Abstract

Objective: This study aims to observe the physiological and pathological changes of severed fingers (limbs) under different storage conditions through animal experiments, and to screen out the best preservation conditions.

Medthods: Sixty healthy adult male Sprague-Dawley rats were selected and evenly divided into 4 preservation groups, including conventional low-temperature dry (CLTD), the university of wisconsin (UW) solution, cryopreservation and cryopreservation + UW solution preservation group. After harvesting the limbs, were preservated for 72 h and 7 days, respectively. Then the limbs were thawed and replanted in situ. Sciatic nerves were collected for hematoxylin and eosin (HE) staining, and observed the changes in tissue morphology.

Results: Replantation was successful in 11 out of 15 rats (73%) in cryopreservation + UW group, and the walking function of the 9 (82%) rats in cryopreservation + UW group were significantly better than that of the cryopreservation preservation group. In addition, the HE staining results shown that the CLTD group nerve bundles were morphologically damaged, and there were more acellular structures and tissue fragments; the UW group nerve bundles were less injured and the perineurium was more complete and orderly; The nerve bundles in the cryopreservation group and cryopreservation + UW group are tightly arranged and the tissue morphology is regular; Compared with the cryopreservation + UW group, the complete of the cryopreservation group is not well.

Conclusions: The cryopreservation technology combined with UW solution is a new and effective method for the severed limbs preserving.

Introduction

Replantation is the surgical reattachment by microsurgical techniques of a body part (finger, hand, or an arm) that has been completely cut from the body. With the continuous advancement of clinical surgical techniques, the replantation of various anatomical parts has become possible [1, 2]. As the opportunities for replanting increase, it becomes more and more important to preserve the body parts for longer and in better conditions. To date, studies have shown that through experiments on preservation and retransplantation of rat limbs, it has been found that the cold ischemic time of limbs should not exceed 12–24 h [3]. Although, in subsequent experiments, studies have found that after wrapping the limb with saline-moistened gauze, sealing it in a plastic bag, and keeping it on ice for 94 h, the finger can still be successfully replanted. However, in order to ensure the success of the operation, it is still recommended that the separated limbs be retransplanted within 12–24 h [4, 5].

The development of cryopreservation technology began when the British biologist Christopher Polge discovered in 1949 that sperm treated with glycerol can survive cryopreservation, and it has been widely used in clinical medicine [6]. Subsequently, Jason G et al. [7] revealed that the ovarian cortical tissue strips of human were preserved using cryopreservation technology, and was transplanted with successful

reproductive function after thawing. Yin et al. [8] reported that the rat ovaries were successful cryopreservation and replantation, and not disrupt the function of the ovaries. In addition, Tanaka et al. [9] reported that the cryopreserved tracheal allografts was successful transplanted in rabbit models. Therefore, it further clarified that the limbs can survive after cryopreservation in vitro. Low-temperature storage can be divided into two categories: one is conventional low-temperature storage at a storage temperature of 4 °C, and the other is deep-low temperature storage, that is, storage in a 80 °C low-temperature refrigerator or liquid nitrogen (-196 °C) [10–12]. Generally, cryoprotectants are added to preserve the limbs while cryopreserving to maintain the biological integrity of the tissues. Cryopreservation is usually used to preserve homogeneous cell populations and monolayer tissues [13]. The purpose of cryopreservation is to keep the tissue in a suspended state, in which the biological process is suspended, and the final temperature is usually the temperature of liquid nitrogen. In addition, bones can be frozen and stored without using cryoprotectants, usually at a temperature not lower than −80 °C [14]. Although various types of tissues can be cryopreserved, the preserved specimens can be thawed and the physiological functions of the limbs can be restored, progress is being made in the cryopreservation of whole organs [14]. However, the optimal parameters for cryopreservation vary for different tissue types [15]. Composite tissues (such as limbs) are composed of multiple tissue types, including skin, muscles, blood vessels, nerves, and bones. Therefore, although cryopreservation of composite tissue is technically challenging, it is still expected to improve the quality of life of many people [16, 17].

Therefore, the purpose of this study is to provide experimental basis for exploring suitable storage methods for amputated fingers (limbs), the effects of rat limbs preserved for 72 h and 7 days under different storage conditions on the survival of limbs after transplantation was observed. Provide methodological reference and corresponding theoretical support for future clinical practice, and provide more guidance cases for the establishment of a limb bank and completion of allogeneic limb replantation in the future, which can more effectively reduce the disability rate of patients and replant the original limbs.

Materials And Methods

Experimental animals and grouping

All animals in this study were cared for, and experiments were performed according to, established guidelines for the use and care of laboratory animals. This study was approved by the Institutional Review Board and the Animal Care and Use Committee of our institution, The First Hospital of Qinhuangdao City. 60 healthy adult male Sprague-Dawley rats approximately five months of age and weighting 200 to 220 g were selected. Animals were obtained from Experimental Animal Center of the First Hospital of Qinhuangdao City, and housed in a 12 hour light/dark cycle and fed standard rat chow and water ad libitum. The 60 rats were divided evenly into conventional low-temperature dry (CLTD) preservation group (n = 15), the university of wisconsin (UW) solution preservation group (n = 15),

cryopreservation preservation group (n = 15) and cryopreservation + UW solution preservation group (n = 15).

Preparation of rat model of severed limb ischemia

The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. The rat was fixed on the operating table, and the bilateral thighs of rats were carried out skin preparation. Then, a curved incision was made at the groin of rat, the subcutaneous tissue, as well as the femoral artery and vein were separated. The ipsilateral circumflex iliac artery and vein, and the pudendal artery and vein were ligated and severed. The hip joint was dissected at the femoral head, and the femoral artery and vein were punctured and cannulated with the indwelling vein, and the tubing was tied firmly. Then, heparin saline was injected into the femoral artery for anticoagulation protection. After the lower limbs were completely dissected, the rat's lower limb skin was sutured continuously from the distal end to the proximal end, and the skeletal muscle tissue was wrapped to reduce the exposed area, avoid infection and excessive evaporation.

Storage of severed limbs in rats

In this study, the 60 rats were divided four groups according to different storage conditions. The storage conditions of the severed limbs of rats in each group are as follows: 1) CLTD preservation group: After the rat limbs were covered with multi-layer oil gauze and were wrapped with a sterile dressing, then put into sterile rubber gloves filled with oxygen, and stored in the refrigerator at a storage temperature of 0–4 °C. 2) UW solution preservation group: The rat limbs were put into UW preservation solution, wrapped in sterile dressing. Then, put into a sterile rubber glove, which was filled with oxygen. Finally, the rat limbs were stored in the refrigerator at a storage temperature of 0–4 °C. 3) Cryopreservation preservation group: After the rat limbs were covered with multi-layer oil gauze and were wrapped with a sterile dressing, then put into sterile rubber gloves filled with oxygen. The two-step storage method was adopted to store the severed limbs of rats (the intermediate temperature is − 20 °C for 60 minutes and then transferred to liquid nitrogen (–196 °C) for storage). 4) Cryopreservation + UW solution preservation group: The rat limbs were put into UW preservation solution, wrapped in sterile dressing, and finally put into sterile rubber gloves, which were filled with oxygen. The two-step storage method was used to store the severed limbs of rats (the intermediate temperature is -20 °C for 60 minutes and then transferred to − 196 °C liquid nitrogen for storage). In addition, the 37 °C warm water bath was used for rewarming in the next experiment.

Replantation

In the four groups, replantation was carried out after the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate and fixed on the operating table, and the skin preparations on both thighs of the rats were performed. After the original incision near the stump, the femur or tibia was exposed, the tibia and femoral arteries and veins of the rats were anastomosed. Finally, the skin is loosely stitched. The left limb was transplanted immediately as the normal group, and for the right limb, transplantation

was performed 7 days after the limb was cooled and preserved. The survival of the severed limb was observed at 72 h and 7 days after replantation.

Hematoxylin-Eosin (HE) staining method to observe the survival of limbs

After replantation, five rats in each group were randomly selected concomitant to nerve harvesting. Muscle samples were fixed in paraformaldehyde, embedded in paraffin, and sectioned in a transverse manner. Sections were then stained with HE. For each sample, images were obtained from three random fields were observed by Olympus BX 51 (Olymupus, Tokyo, Japan).

Results

General observation

Replantation was successful in 6 out of 15 rats (40%) in CLTD group, 8 out of 15 rats (53%) in UW group, 9 out of 15 rats (60%) in cryopreservation group, and 11 out of 15 rats (73%) in cryopreservation + UW group. The animals started to eat food on the first day after retransplantation; the incision healed well at 1 week, and no infection occurred. The 6 rats in CLTD group and 8 rats in UW group have toe paralysis, limited opening, and muscle swelling. Among them, the UW group has less paralysis, toe contracture and muscle atrophy than the CLTD group, and there are 4 rats that walking function is better than that of the CLTD group. The 9 rats and 11 rats were transplanted successfully that in cryopreservation group and cryopreservation + UW group, respectively, which had no obvious abnormalities in the lower limbs, and the walking function was normal, and the walking function of the 9 (82%) rats in cryopreservation + UW group were significantly better than that of the cryopreservation preservation group.

Histological observation

The HE staining of each group was shown in Fig. 1. Compared with the normal group, the CLTD group nerve bundles were morphologically damaged, the perineurium was irregular, and there were more acellular structures and tissue fragments. In addition, the tissues of the 7 days preservation group were significantly damaged more serious than those of the 72 h preservation group, revealing that the preservation of limbs by the CLTD method will prolong the limbs over time, and the limb injuries will be more serious, which is not conducive to the functional recovery of the limbs after transplantation. Compared with the CLTD group, the UW group nerve bundles were less injured and the perineurium was more complete and orderly; The nerve bundles in the cryopreservation group and cryopreservation + UW group are tightly arranged and the perineum morphology is regular; Compared with the cryopreservation + UW group, the complete of the cryopreservation group is not well, and in cryopreservation + UW group, there is no significant difference between the 72 h group and the 7days group. Therefore, cryopreservation + UW solution can extend the preservation time of limbs and the function of the limbs was better restored after transplantation.

Discussions

Replantation is the best choice for severed fingers [18, 19]. However, replantation would be delayed or cancelled when the patient has serious organ damage or wound pollution, so that the severed fingers will be discarded. The lives of patients will be greatly troubled [20]. The ectopic replantation of severed fingers faces great challenges [21]. Surgeons and patients are looking for long-term preservation of severed fingers. Cryopreservation technology have been used in the preservation of human cells, embryos and ovaries for a long time, and have achieved good clinical results [22, 23]. Especially, patients with cryopreserved ovarian tissue transplantation have pregnancy and live birth. In 2004, study shown that the cryopreserved finger was successfully transplanted and survived, provide useful information on finger long-term preservation [24]. There is no progress in cryopreservation of large composite tissues in recent years, partly because uniform freezing and thawing cannot be achieved, which is the key step of tissue survive [2].

At present, the research on cryopreservation of simple tissue blocks has made phased progress, but there are few researches on cryopreservation of complex tissue limbs [25]. With the rapid development of microsurgery technology and the strong desire of patients to save limbs. The cryopreservation technology is an important preservation method for preservation of limbs, providing good basic support for the establishment of the body bank in the future [26].

In this study, we verified the protective effects of CLTD, the UW solution, cryopreservation and cryopreservation + UW solution on limbs in rats, respectively. Although the decline in muscle mass, but the function of the limbs after transplantation was restored to a certain extent, especially the limbs was well recovered by cryopreservation + UW solution preserved. These results show that cryopreservation + UW solution can extend the preservation time of limbs. Moreover, the cryopreservation technology combined with UW solution is the first time was used to preserve rat severed limbs, and the function of the limbs was better restored after transplantation. Our study may provide experimental basis for the preservation method of severed fingers and create theoretical conditions for the physical operation, and provide the possibility of limb survival for patients who are willing to replant.

Conclusion

In summary, this study successfully completed the replantation of rat limbs, revealing that the cryopreservation technology combined with UW solution is a new and effective method for the severed limbs preserving, which may provide useful information for cryopreservation of human organs and composite tissues in the future.

Declarations

Ethics approval and consent to participate

Approval was obtained from the Institutional Review Board and the Animal Care and Use Committee of our institution, the First Hospital of Qinhuangdao City. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent for publication

All authors of this paper consent for publishing manuscript and figures in the Journal.

Conflict of interest

The authors declared that this article does not have any conflict of interest.

Availability of data and material

The data and materials are available under the permission of author.

Competing interests

The author declares that they have no competing interests.

Funding

The authors received no financial support for this study.

Acknowledgements

Not applicable.

Authors' contributions

Yu Tian and Nan Li conceived, designed and priformed the experiments. Wei Wang analyzed and interpreted the data. Na Li contributed methods, materials, analysis tools or data. Yu Tian wrote the paper. All authors read and approved the final manuscript.

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Figures

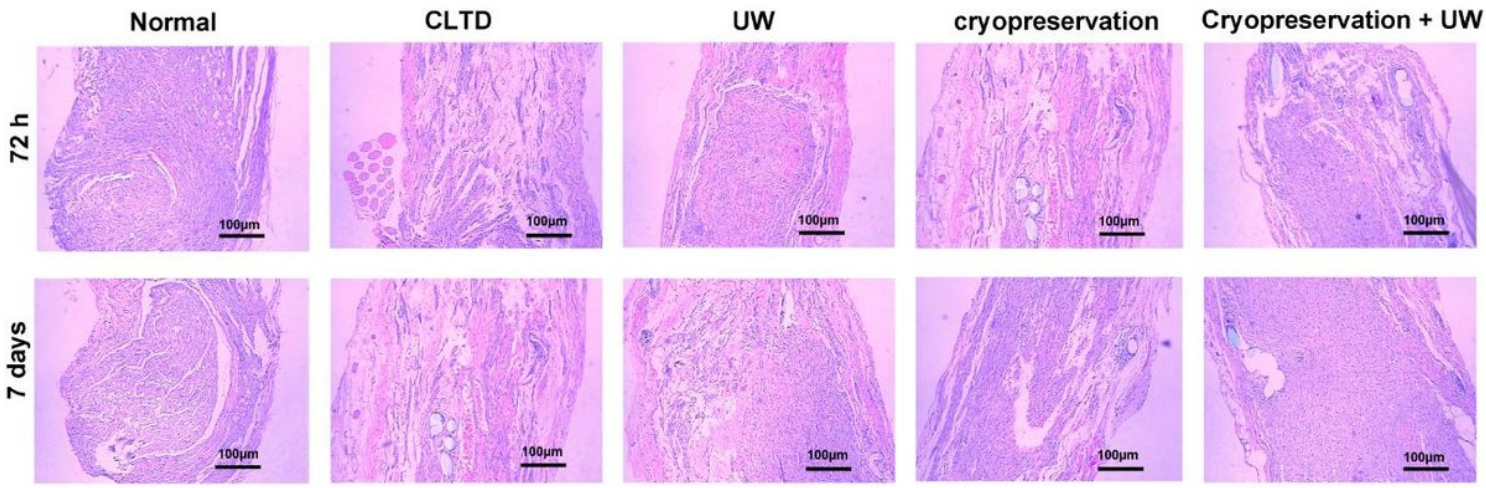

Figure 1

Hematoxylin-Eosin (HE) staining method to observe the survival of limbs, CLTD, the conventional low-temperature dry preservation group; UW, the university of wisconsin solution preservation group;