Research Progress of Limb Preservation Methods: A Review
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Abstract
The effects of the survival rate of replanted limbs and postoperative functional recovery is closely related to the correctness and rationality of the limb preservation methods. However, the limb preservation methods can extend the activity of tissues and cells in different levels, reduce ischemia-reperfusion injury and improve the replant survival rate and functional recovery. Is it important to consider the injury situation, the environment and the requirements of replanting of limbs, how to preserve organically and replant reasonably, and to develop strengths and avoid weaknesses of various methods and techniques that are most suitable for limb preservation? This review describes the research progress and analyzes the effects of different methods of limb preservation.

Keywords: Limb Preservation, Low-Temperature, Perfusion, Reperfusion Injury, Hyperbaric Oxygen.

INTRODUCTION
In clinical practice, what affects the survival rate of replanted limbs and postoperative functional recovery is not only related to the level of microsurgery techniques, but also closely related to the correctness and rationality of the preservation methods of the limbs. Many studies have shown that the main factors affecting the survival rate and functional recovery of severed limb reimplantation are ischemia and hypoxia injury and reperfusion injury after circulatory recanalization.1-6 For the severed limb, there are obvious differences in the tolerance of different tissues to ischemia and hypoxia. At room temperature, the worst tolerance is muscle tissue and blood vessels, followed by nerves, fat, and skin, while the strongest tolerance is bone tissue. 7 When the limb is severed, the blood circulation will stop immediately, various metabolism and oxygen supply will be suspended, and metabolites will accumulate, resulting in changes in physiology, tissue morphology, and ultrastructure, such as cytoplasmic vacuole formation, acidosis, calcium overload, mitochondrial edema, energy consumption, etc., and reperfusion after circulation reconstruction will further aggravate tissue injury.8,9

The key to reducing those injuries is to rebuild the blood circulation as soon as possible, shorten the ischemic time, and reasonably preserve the limbs.10 How to reasonably and effectively preserve the severed limb before the operation, reduce the tissue damage caused by ischemia and hypoxia, delay the metabolic process, provide energy for the severed tissue, remove the accumulation of metabolic and toxic products in the tissue, and prolong the degeneration and necrosis of tissue cells has become particularly key.11 It has become one of the further research directions of reimplantation surgery to correctly and reasonably preserve the limbs, save time for reimplantation surgery, reduce ischemia-reperfusion injury after replantation, and improve reimplantation survival rate and postoperative functional recovery.12,13

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In this review, we focus on the wide-scale implementation of limb preservation methods that enable prolonged safe preservation and survival rate of severed limbs.

**Low-Temperature Dry and Cold Storage Method**

Low temperature can effectively reduce the metabolic rate of cell tissue and reduce the damage of intracellular enzymes to cell tissue. All chemical reactions in the process of metabolism in cells are inhibited by low temperature. When the temperature drops to a certain extent, all chemical changes in cells are in a "suspended" state. In the 1930s, Allen first studied the effect of temperature on ischemic tolerance of severed limbs, found that low temperature could effectively reduce tissue metabolic activity, and pointed out that the optimal temperature should be the lowest temperature without tissue damage. The method of dry and low-temperature preservation is recommended, that is, wrap the broken limb with gauze, put it in plastic bag for sealing, and then put it into ice water for drying and refrigeration. This method has been used until now. In a rabbit experiment, it is concluded from the cell structure level that there is an inverse correlation between the preservation temperature and time of tissue. The lower the temperature is related with the longer the time for muscle tissue lesions and necrosis. A study of rat hindlimb ischemia-reperfusion injury model with a rubber tourniquet established that low temperature and ozone had a protective effect on rat skeletal muscle ischemia-reperfusion injury, but there was no synergistic effect between them.

It should be emphasized that low temperature can reduce the activity of enzymes in tissues and cells, so as to delay the destruction speed of tissues and cells and reduce cell metabolic rate and energy consumption. However, low temperature can only reduce the speed of various metabolic reactions in cells, delay the process of tissue cell degeneration and necrosis, but can not stop tissue cell metabolism.

**Cryopreservation**

Deep cryopreservation is a method of long-term preservation with the help of an extremely low temperature (-196°C) environment to make the energy metabolism of biological tissues close to stop and prolong their biological activity. After rewarming under appropriate conditions, biological tissues can restore their metabolic activity and biological function. At present, the method is mainly divided into two types: slow freezing method and vitrification freezing method. The slow freezing method is divided into program controlled cooling and gradient cooling, etc. The rewarming method is mainly fast rewarming method and slow rewarming method. The cryoprotectants used include hydroxyl-containing compounds (such as polyols, amino alcohols, trehalose, etc.), methyl-containing compounds (such as dimethyl sulfoxide, dimethylformamide, etc.), as well as both hydroxyl and Compounds containing methyl groups (such as propylene glycol, butylene glycol and their isomers).

In transplantation, repair and reconstruction surgery, cryopreservation of various biological tissues such as blood vessels, nerves and bones is deeply studied and widely used. In a vascular cryopreservation experiment, 15% Dimethyl sulfoxide (DMSO) protective solution was used to cryopreserve blood vessels. The results showed that 70% of the ultrastructure of vascular tissue was well preserved and the function of blood vessels was well maintained.

In addition, the effects of different periods of ischemia on the ultrastructure of bone and blood vessels in rats were studied. The tissues were cryopreserved in liquid nitrogen. The results showed that the changes of vascular endothelial cells were slight after 8 hours of ischemia, but there was no significant change of bone cells. In the study of peripheral nerve cryopreservation effectively protected the activity of nerve tissue after deep hypothermia preservation of rat sciatic nerve. What is more, there are animal experiments whereas clinical application have proven that the bone healing time after deep hypothermic refrigeration is basically the same as that of vascularized autologous bone transplantation.

At present, deep cryopreservation has been deeply studied, but most of them stay in the freezing rewarming stage of single cells or tissues. However, a clinical observation reported in 2004 showed that a severed index finger was frozen by programmed freezing method and stored in -196°C liquid nitrogen. After 81 days of preservation, the replantation was successful, and the sensory function of the replanted finger was good. This report greatly stimulated people's research on deep cryopreservation composite tissue.
Deep cryopreservation of severed limbs is a multi-cell freezing process of composite tissue. Each cell has different requirements for the type, concentration and freezing rewarming rate of cryoprotectant. What concentration of cryoprotectant is selected, how to ensure the uniform cooling and rewarming of composite tissue as a whole, and how to avoid the damage caused by different thermal expansion and cold contraction of each part caused by the temperature gradient around and in the central area of the composite tissue are problems that still need to be further explored.

**Hypothermic Perfusion Preservation**

In organ transplantation surgery, organ preservation solutions such as the University of Wisconsin (UW) solution, histidine-tryptophan-ketoglutarate (HTK) solution, euor Collins solution and Celsior solution are widely used to preserve transplanted organs at low temperature.32,33 Similarly, many scholars have studied that on the basis of low temperature cold preservation, a variety of perfusion solutions are used to perfuse the severed limbs, which correspondingly prolongs the time limit of limb replantation, including organ preservation solution.34-36 Free radical scavenger perfusion solution, energy mixture perfusion solution, blood substitutes and whole blood, etc.

During the perfusion of limbs with perfusion solution, the degree of tissue vascular bed injury can be understood, the vasospasm can be relieved, the vascular lumen can be expanded, the absorption function of capillaries can be restored, and the basic energy substances required for metabolism can be provided for the tissue through perfusion, so that the tissue can maintain a low metabolic state. It is conducive to maintain the stability of cell membrane, effectively flush out the metabolites such as lactic acid stored in tissues and the residual blood and blood clots in blood vessels through perfusion, reduce the absorption of metabolites after replantation, reduce the generation of harmful components such as oxygen free radicals, and reduce intracellular acidosis and ischemia-reperfusion injury.37

In the study, UW solution was used to perfusion Wistar rats' severed hind limbs at 4 degree temperature. It was found that compared with the control group, the muscle fibers of the perfusion group were lighter and less broken, and the cells had less transverse striation. It was proved that the UW solution had better effect on Cryopreservation of the limb after perfusion.38 The experiment of perfusing the severed hindlimb of Wistar rats with UW solution at 4℃ found that compared with the control group, the perfusion group had less muscle fiber edema, less fracture, fewer cells and transverse striation dissolution, which proved that the effect of low-temperature preservation after perfusing the severed limb with UW solution was better.20

During ischemia and reperfusion, tissues and organs produce a large number of O-2, H2O2 and oxygen free radicals such as OH-, which damage lipids (including biofilms), proteins (including enzymes, structural and functional proteins) and nucleic acids. In many experimental studies of ischemia-reperfusion injury, pre perfusion treatment with free radical scavengers can well reduce ischemia-reperfusion injury.39 Free radical scavenger perfusion solutions mainly include low molecular scavengers and enzyme scavengers. Among them, low molecular scavengers include fat soluble Vitamin E and Vitamin A, cysteine, Vitamin C, reduced glutathione (GSH) and NADPH, and enzymatic scavengers include intracellular catalase (CAT), peroxidase (such as GSHPx) and superoxide dismutase (SOD).40 Studies have been designed to supplement four hydrogen biopterin, L-arginine and Vitamin C to the hind limb ischemia model in rats, thereby increasing the biological activity of nitric oxide, reducing oxidative stress, promoting collateral circulation formation and reducing muscle necrosis, and improving blood flow recovery after hind limb ischemia in rats.1 In the study of the effect of superoxide dismutase (SOD) perfusion on the ultrastructure of isolated vascular smooth muscle in rats, the severed limbs of rats were perfused with SOD and normal saline and stored at 4℃ to observe the ultrastructural changes of vascular smooth muscle. After SOD perfusion, the ultrastructural changes of femoral artery vascular smooth muscle were significantly delayed and had a protective effect on vascular smooth muscle.41 The study confirmed that energy mixture can provide energy for Na+-K+-ATP enzymatic activity in skeletal muscle and maintain its activity under ischemic conditions for 2 ~ 4 hours; An appropriate amount of hormone contained in energy mixture has a series of anti ischemia-reperfusion effects.42
Many scholars use blood substitutes to perfuse the severed limbs, so as to reduce tissue ischemia and hypoxia injury and reperfusion injury, and achieve the purpose of effectively preserving the limbs. Araki et al. developed an artificial oxygen carrying hemoglobin vesicle (HbVs). The severed limbs of rats were perfused with ETK solution and HbVs solution for 6 hours and preserved at room temperature for replantation. The results showed that the oxygen cooperation of the severed limbs perfused with HbVs was better than that perfused with ETK solution alone, and the gastrocnemius muscle was well preserved, which was conducive to the survival rate of replantation.6

In organ transplantation surgery, many scholars have studied the effect of extracorporeal circulation whole blood perfusion on the preservation of isolated liver, kidney and lung. Similarly, the preservation of the 12h of pigs' severed forelimbs by extracorporeal circulation and autologous blood perfusion was compared with that of the simple cold storage control group. The results showed that the pH value of the tissue was stable after ischemia and reperfusion, and the damage of ischemia and hypoxia was mild, which was effective for the protection of the severed limb.43 In the study of New Zealand sheep's severed limbs in vitro, the same type of blood perfusion test was carried out. It was found that decreasing oxygen volume fraction can make the arterial oxygen partial pressure close to the normal range, thereby reducing the production of oxygen free radicals, reducing the release of creatine kinase, lactate dehydrogenase and troponin, which is beneficial to the protection of limb muscles.44 The limbs of Bama miniature pig were cultured in vitro at different temperatures to simulate the physiological environment in vivo. The results showed that in vitro blood perfusion at 10°C simulated the physiological environment in vivo, and foster amputated limbs could significantly inhibit the injury of skeletal muscle cells caused by reperfusion.45

After perfusing the severed limbs with the above-mentioned perfusion solutions, different preservation effects were obtained by improving capillary permeability, reducing cell edema, reducing cell membrane permeability, inhibiting the production of oxygen free radicals and reducing the formation of lipid peroxide. However, the specific component of perfusion solution most suitable for the preservation of limb composite tissue, and many other problems need to be further discussed and solved, such as what kind of perfusion method should be applied, how to master the perfusion pressure and perfusion speed, and the best preservation time after perfusion.

Hyperbaric Oxygen Preservation

At present, hyperbaric oxygen (HBO) is widely used in various fields of medicine as a treatment of hypoxia related diseases with high pressure and high concentration oxygen. HBO can improve tissue oxygen solubility and reduce oxygen consumption by increasing blood oxygen partial pressure, improve tissue oxygen dispersion and distance, make cells obtain enough oxygen, which is conducive to the recovery of glucose metabolism and energy supply, reduce the accumulation of metabolites produced by hypoxia, promote the regeneration of capillaries and the establishment of collateral circulation, improve microcirculation, reduce tissue edema and promote the functional recovery of damaged tissues and cells. In recent years, many scholars have studied the effect of hyperbaric oxygen on the preservation of severed limbs, and found that hyperbaric oxygen can improve the survival rate of replantation of severed limbs.46

For example, studies have confirmed that hyperbaric oxygen can effectively reduce ischemia-reperfusion injury of skeletal muscle and other tissues through nitric oxide synthase.47 In the study of the effect of hyperbaric oxygen on the replantation of severed limbs at room temperature for a long time, the severed limb model of rats was used, which was preserved at room temperature for 4h and replanted. After operation, hyperbaric oxygen adjuvant treatment was given and comparative study was carried out. Considering the poor prognosis, the replantation of severed limbs at extended room temperature was considered as a relative contraindication, but the results showed that if postoperative adjuvant hyperbaric oxygen therapy is performed, the indications for replantation can be relaxed.48 In China, in the experimental study of the protective effect of HBO Preservation on severed limbs, the rat hindlimb amputation model was stored in HBO and indoor air respectively. The enzyme activity of skeletal muscle and the ultrastructural changes of skeletal muscle were measured. The serum enzyme content was measured after replantation. The limb survival rate was evaluated on the 10th day after operation. The results showed that the changes of enzyme activity and ultrastructure in
HBO were better than those in indoor air, indicating that hyperbaric oxygen preservation had a protective effect on the severed limbs of rats.28

**Ectopic Foster Preservation Method**

In clinical practice, we often encounter some special and difficult limb amputation injuries. For example, limb amputation caused by trauma is often combined with serious injury of other organs. We should consider saving lives first and can not tolerate long-term replantation, or the severed end of the severed limb is widely damaged, so the primary replantation is difficult to succeed. In this regard, some scholars temporarily transplant the severed limb to other parts of the body, and then perform in situ replantation when conditions permit. Two cases 49,50 of ectopic foster replantation of severed limbs were reported. One was explosive injury of the right hand. The right thumb was temporarily placed in the radial artery of the ipsilateral wrist through end-to-side anastomosis, and replantation was successful 7 days later; Another case was severe avulsion injury of the right forearm and palm. The index finger and little finger were ectopic fostered at the radial artery of the opposite forearm, but the index finger could not be saved due to the heavy damage. After 9 days, the little finger was replanted back to the original ring finger.51

Another report is a case of transplanting a right severed wrist to the stump of the left forelimb. The patient had amputation of the upper limb in varying degrees, extensive soft tissue and bone injury at the distal end of the right side, and destructive injury from the end of the finger including the thumb to the metacarpophalangeal joint on the left side. Therefore, the right hand was transplanted to the left hand to reconstruct the distal function of the limb. Six months after operation, it was found that it had good motor function of wrist, thumb and finger. It has palmar function, but the function of finger internal muscle is poor, including external rotation and adduction. The Tinel sign of the distal phalanx of the middle finger and the distal phalanx of the distal phalanx of the little finger is positive. The patients can complete most of their daily activities and are satisfied with the recovery of function.52

In China, it has been reported that the severed forearm with serious injury is treated by one-stage ectopic foster care, two-stage in-situ replantation and three-stage functional reconstruction and repair, so that the severed limb can survive and restore part of the feeling and function, which provides a feasible method to save the complex severed limb.53 However, this method has more complex operations, more time and higher risk, The recovery of limb function and sensation after replantation is poor. Therefore, the indications should be strictly mastered.54 Although the method of ectopic foster, preservation and replanting has solved many of the first broken limbs, the limbs have undergone two replantation operations and the functional recovery may be slightly worse.

**CONCLUSION**

Due to the complex tissue structure of the limb, different separation planes, different environments of the severed limb and other factors, there is no satisfactory preservation method at present. The above mentioned methods have their own pros and cons. The low-temperature drying and refrigeration preservation method is economical, simple and easy to operate, but the preservation time is short. The time limit of replantation of severed limbs was also prolonged by various perfusion liquid perfusion and low-temperature preservation methods, but the problems of perfusion method, pressure, speed and time need to be studied and solved.

Although the whole blood perfusion and preservation method of extracorporeal circulation has a good protective effect on the broken limbs, the application of whole blood may cause secondary injury to itself, and the operation of extracorporeal circulation is cumbersome and can not be applied in time. Deep cryopreservation has a good effect on tissue long-term preservation, but cryoprotectants will cause damage to cells in the process of cooling and rewarming, and also have certain cytotoxicity. Hyperbaric oxygen preservation has a certain protective effect on the severed limbs, but it requires higher storage and special containers.

Lastly, the best method of limb preservation should be based on prolonging tissue cell activity and improving replantation survival rate and functional recovery.
Declaration of Conflicting Interests

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