

Received: 2019.05.27

Accepted: 2019.07.04

Published: 2019.10.22

Dimethyl Sulfoxide is Less Effective in Immersing Cryopreserved Large Pieces of Tissue: A Rabbit Hind-Limb Model

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: This study was Supported by the Supporting Program of the "Eleventh Five-year Plan" for Sci & Tech Research of China (08G051)

Background: Dimethyl sulfoxide (DMSO) cryoprotectant can effectively alleviate the damage to single tissue during cryopreservation and restore its physiological activity after rewarming. However, studies have not been successful for preserving large tissue. This study aimed to investigate the application conditions of DMSO in large composite-tissue by performing femoral artery perfusion and soaking in a rabbit hind-limb model.

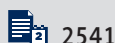
Material/Methods: A microdialysis-freezing point osmometer was used to detect the minimum time required for effective perfusion of 10% v/v perfusion and 20% v/v perfusion group. Magnetic resonance spectroscopy (MRS) was used to detect the area under the spectrum peak of DMSO in perivascular, intramuscular, subcutaneous areas, and compare the area under the spectrum peak in the 20% vascular perfusion group and other whole immersion groups.

Results: The minimum time required for effective perfusion of muscle in the 10% v/v group was 30 minutes, the DMSO concentration was stable at 5% v/v; In the 20% v/v group the minimum time was at 20 minutes, stable at 12% v/v. There was a statistically difference of the area under the spectrum peak in the 10% group and the 20% v/v group after vascular perfusion in different tissue locations ($P < 0.05$). The 20% vascular perfusion group and the different concentration of DMSO in the large tissue soaking group were statistically different ($P < 0.05$). There was a significant difference in the 20% v/v vascular perfusion group compared to the low concentration immersion group, but no difference compared to the 50% immersion group.

Conclusions: The effect of blood perfusion on cryopreservation in large tissue by using DMSO was slightly better than overall soaking, especially in preservation of skin and subcutaneous tissue.

MeSH Keywords: Composite Tissue Allografts • Cottontail Rabbit Papillomavirus • Cryopreservation • Dimethyl Sulfoxide

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/917775>



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Background

Dimethyl sulfoxide (DMSO) is one of the most commonly used cryogenic protectants at present. Biological tissues and cells can be effectively vitrified when soaked with 10% DMSO. In recent years, DMSO has been widely used as a cryoprotectant for cryopreservation of various tissues and organs of animals and plants. For example, DMSO is used for cryopreservation of pig oocytes and embryos, mouse testes and ovaries, and sperm of various fish and other animals. At present, DMSO is also applied to human skin [1], mesenchymal stem cells [2], human chondrocytes [3,4], fatty liver cells [5], and articular cartilage [6]. Thus, it can be effective in reducing the damage of cryopreservation and restoring most of the physiological activity after rewarming for biological tissues. However, it is unsatisfactory to preserve various types of large composite tissues when using the same method (10% DMSO) [7]. In the Wang Xiao-tong study [8], 18 human ovarian tissues were processed by resection, and slicing and freezing the tissues (each piece was 18×10×1 mm). The experiment was divided into 3 groups: 1) vitrification group, 2) programmed frozen group, and 3) fresh control group. The results showed that vitrification had certain advantages in preserving ovarian interstitial cells, thus suggesting that vitrification of large ovarian tissue in the human body is feasible.

Kim [9] assessment of long-term endocrine function after transplantation of frozen-thawed human ovarian tissue to the heterotopic site through 10-year longitudinal follow-up study. Their study showed that long-term endocrine function lasting for 7 years can be established with heterotopic transplantation of cryobanked human ovarian tissue-thin slices (5×5×1 mm). However, it is unknown whether large complex tissue has the effective protective agent concentration required to achieve cell vitrification like a single tissue or cell, especially when whole immersing or arterial infusing after in DMSO.

Therefore, in this study, we will quantitatively evaluate the concentration of DMSO in whole immersion and arterial infusion of bulk composite tissues, and to explore the optimal preservation conditions of DMSO for large composite tissues.

Material and Methods

Disconnect of rabbit hind-limbs model

Fifty healthy New Zealand white rabbits, weighing 2.5 to 3.0 kg, were provided by Beijing Agricultural College; the animal license number of SYXK (Jing) 2015-0004. Healthy adult New Zealand white rabbits were sacrificed by ear vein air embolization. The rabbit hind limbs were removed from the hips, and thus we obtained the hind-limb amputation model. This study

was examined and approved by our unit's animal ethics experiment. In this study, a composite tissue sample was defined as a diameter greater than 1.5 cm and intact skin was defined as a large composite tissue.

Study procedure

Minimum effective perfusion time

Sixteen rabbits from a hind-limb amputation model were randomly divided into a 10% v/v perfusion group (A1 group) and a 20% v/v perfusion group (A2 group), with 8 rabbits in each group. The microdialysis probes were pre-placed in the model muscle tissue and perfused continuously through the femoral artery with 10% v/v and 20% v/v concentrations of DMSO, respectively. And then, the samples were taken at 1, 3, 5, 10, 20, 30, 40, and 50 minutes. The exact concentration of dialyzed DMSO was measured using a freezing point osmometer, and the time-concentration curve was plotted to compare the characteristics of these 2 perfusion groups.

The arterial infusion groups

The other 16 rabbits were randomly divided into a 10% v/v vascular perfusion group (B1 group) and a 20% v/v vascular perfusion group (B2 group), with 8 rabbits in each group. After continuous infusion of 10% v/v DMSO through the femoral artery for 30 minutes in group B1, MRS was used to detect the peak area under the perivascular, intramuscular, and subcutaneous DMSO areas in the isolated hind-limb model of the rabbit. The B2 group was continuously perfused with 20%v/v concentration of DMSO into the femoral artery. After 20 minutes, MRS was used to detect the peak area under the curve of DMSO concentration after vascular perfusion of the perivascular, intramuscular, and subcutaneous areas in the isolated hind-limb model. Test results were used to compare the different tissue sites in these 2 DMSO concentration vascular perfusion groups.

The whole immersion groups

Eighteen rabbits were randomly divided into a 50% v/v soaking group (C1 group), a 35% v/v group (C2 group), and a 20% v/v group (C3 group), with 6 rabbits in each group. The disconnected of skin in the hind-limb models was reserved sufficiently in the closed section. Each sample was immersed in a 50% v/v, a 35% v/v, and a 20% v/v concentration of DMSO for 30 minutes at room temperature. And then, MRS was used to detect perivascular, intramuscular, and subcutaneous areas in each sample model. The area under the peak of DMSO was compared between different distribution groups of model tissues.

Cryoprotectant DMSO

Dimethyl sulfoxide [DMSO, D4540, 99.5% (GC), cell grade, 100 mL: Sigma-Aldrich, Munich, Germany] was diluted with physiological saline to the desired of volume fraction concentration.

Microdialysis and freezing point osmometer

Microdialysis is a sampling technique of biochemical extracellular used to detected liquid biochemical, and it is often used in clinical research, drug testing, and new drug development [10]. The characteristics of microdialysis is screening out the soluble molecules of the local intercellular substance by using a semi-permeable membrane at the tip of the probe, and continuous detection sample at different times [11]. Previous studies have confirmed that microdialysis can obtain a stable relative recovery (RR, the ratio of sample concentration to actual concentration) of DMSO and can be used to measure the average recovery under the study condition [12].

The principle of freezing point osmometer is to use a linear relationship between the osmolality of the solution and the drop in the freezing point temperature to detect the molar concentration of the solution permeation, thereby accurately obtaining the concentration of the solute in the solution. Previous studies have found that microdialysis can be used in conjunction with a freezing point osmometer to quantitatively detect the concentration of a target at the microdialysis tube [13].

Nuclear magnetic resonance spectroscopy (MRS)

Studies have shown that different compounds produced different resonance frequencies in the magnetic field, and a chemical shift occurs. According to this principle, MRS can distinguish different compounds in the frequency axis, and use chemical shift and J-coupling to convert the acquired information into a spectrum, the horizontal axis represents the chemical shift, and the vertical axis peak represents the signal intensity, while the peak of the spectrum The area is proportional to the concentration of the species that produces the peak, which is the basis for structural inference and quantitative analysis of the corresponding compounds by MRS [14]. Although MRS cannot be continuously measured the concentration of the compound, it has the following characteristics: completely non-invasive, accurate positioning, high sensitivity, and quantitative analysis of tissue metabolic changes at the molecular level by imaging technology [15].

We used the US BRUKER 7.0T (Pharma Scan 70/16 US) superconducting magnetic resonance imaging (MRI) system and its own orthogonal coil for small animal detection. The previous experiments proved that DMSO resonates *in vitro* and *in vivo*

in the rabbit hind-limb model. The frequency and the chemical shift are about in the region of 2.6. The MRI plain image can accurately locate and measure the DMSO signal peaks in the subcutaneous and muscle vessels and calculate the area under the peak (Figure 1).

Statistical analysis

SPSS 20.0 (IBM) and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) were used to analysis, and mapping of data. All data in this study were expressed as mean \pm standard deviation (mean \pm SD). Two-sided Student's *t*-test was performed using the same software package. *P* values <0.05 were considered significant.

Results

Minimum effective perfusion time

The A1 group was perfused with DMSO at 10% concentration, and the intramuscular DMSO concentration gradually increased and stabilized at 5% after about 30 minutes. The A2 group was perfused with 20% DMSO concentration, and the intramuscular DMSO concentration gradually increased and stabilized at about 12% after about 20 minutes. The DMSO concentration curves of the two groups at the different time showed Figure 2.

The arterial infusion groups

The other 16 rabbits were randomly divided into a 10% v/v vascular perfusion group (B1 group) and a 20% v/v vascular perfusion group (B2 group), with 8 rabbits in each group. There was a statistically significant difference of DMSO concentration at different sites (vascular, perivascular, and subcutaneous) between the 10% perfusion group and 20% perfusion group after vascular perfusion (All *P* values were <0.05), more details shown in Figure 3.

The whole immersing groups

Eighteen rabbits were randomly divided into a 50% v/v soaking group (C1 group), a 35% v/v group (C2 group), and a 20% v/v group (C3 group), with 6 rabbits in each group. In the macro-tissue immersion of different concentration of DMSO groups, we didn't detect DMSO peaks in the muscles and the blood vessels. The 20% DMSO vascular perfusion group and the different concentration of DMSO in large tissue soaking groups were statistically different (*P*<0.05). However, there was a significant difference between the 20% v/v vascular perfusion group and the 35% immersion group and the 20% immersion group, but not the 50% immersion group (*P*=0.001, *P*=0.001, and *P*=0.196), more details shown in Figure 4.

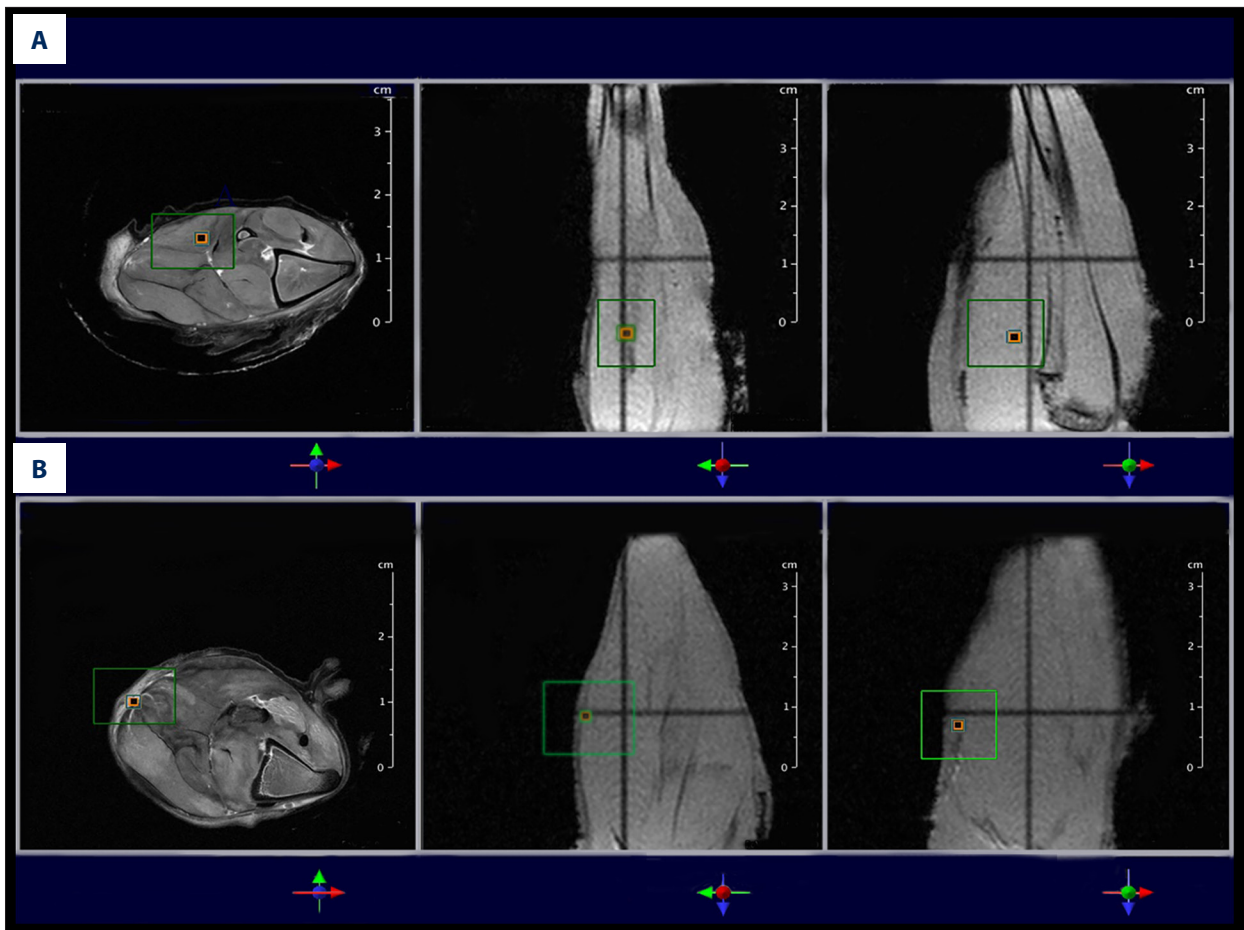


Figure 1. Magnetic resonance spectroscopy detection the different parts of dimethyl sulfoxide concentrations in the target model. (A) Muscle tissues; (B) Subcutaneous tissues. F – frontal position; L – lateral position; A – axial position.

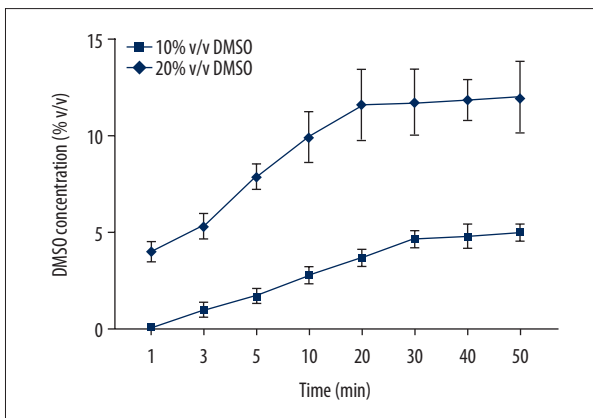


Figure 2. The trend of muscle concentration after vascular perfusion at different time points in the 10% (A1) and the 20% (A2) dimethyl sulfoxide group.

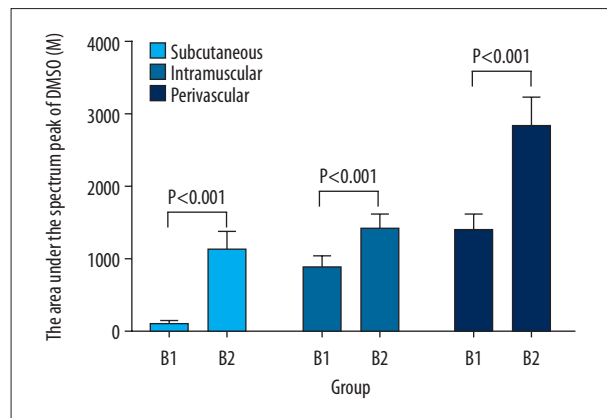


Figure 3. Arterial infusion between in the 10% (B1) and the 20% (B2) dimethyl sulfoxide concentration of the area under the spectrum peak by magnetic resonance spectroscopy detection in different tissue parts (perivascular, intramuscular, and subcutaneous).

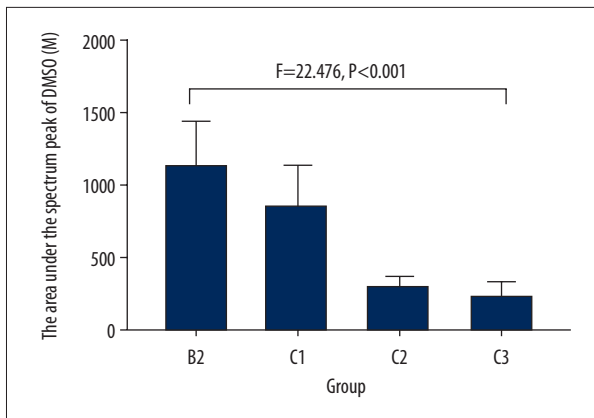


Figure 4. Arterial infusion and whole immersing of the area under the spectrum peak by magnetic resonance spectroscopy detection in different dimethyl sulfoxide (DMSO) concentration group. B2 – arterial infusion in 20% DMSO; C1 – 50% v/v soaking group; C2 – 35% v/v group; C3 – 20% v/v group.

Discussion

With the development of cryopreservation medical technology, the technology of cryopreservation of cells and single tissue has been quite mature in recent studies [16]. However, the preservation of more complex large-sized composite tissues is still in the experimental exploration stage. Cui et al. in a preliminary study of cryopreservation of composite tissue in rabbits, showed that there was a healthy flap color and hair growth until the 63rd day of transplantation when the rabbit was cryopreserved with vascular flap in the abdomen [17]. Their results demonstrated that long term survival of cryopreserved composite tissue transplants is possible. Wang Zheng-tao et al. reported a case of replantation of severed fingers in a patient who had been cryopreserved for 81 days [18]. Although these studies show that the composite tissue is likely to survive after long-term cryopreservation and replantation, the aforementioned studies are all sporadic cases; the vitrification cryopreservation method for large composite tissues has not been clearly established, and stable rewarming survival cannot be obtained [19]. The reason might be that the composite tissue has a thick texture and a complicated structure, and the cryoprotectant cannot smoothly reach around the composite tissue cells and complete vitrification. During freezing and rewarming, the tissue cells form ice crystals that cause irreversible damage to the tissue [20].

DMSO has high permeability, good thermal stability, and is miscible with water, which is commonly used in cryoprotectant. It has low relative molecular weight and can be combined with water at any ratio to efficiently infiltrate into the cell, and the formation of ice crystals can be effectively controlled [21]. When the concentration of DMSO in the biological tissue reaches

10% v/v, cell vitrification can be completed, and cells can be protected from deep low-temperature damage [22]. In our study, rabbit hind-limbs were used as a large composite tissue research model, and the cryoprotectant DMSO was used as the target test substance. The model was studied by immersion and vascular perfusion at the current commonly used DMSO concentration, and the microdialysis-ice point osmometer was passed. NMR spectroscopy was used to detect the concentration of DMSO in local tissues, and to determine the introduction effect of cryoprotectant. The results of this study showed that: 1) the concentration of DMSO in the muscle tissue of the model of perfusion group entered the plateau after a certain period of perfusion, and the increase of DMSO concentration in the muscle tissue continued to be slowed down. Considering that the isolated tissue should minimize the warm ischemia time, the target tissue should reach 10% v/v DMSO concentration before the plateau or plateau. 2) Microdialysis continuously samples the A1 and A2 models on the time axis. It is found that the A1 group enters the plateau period for about 30 minutes, while the A2 group takes about 20 minutes, indicating that increasing the DMSO perfusion concentration can shorten the time to enter the plateau. In turn, the perfusion time is shortened; however, if the perfused DMSO concentration is too high, the vascular endothelium is damaged, which has a devastating effect on re-implantation after rewarming [23,24]. Studies have shown that in order to maintain the cell activity of tissue engineered dermal tissue above 60%, the soaking concentration of the DMSO solution as a protective agent should be less than 20% v/v. Therefore, the highest DMSO concentration selected for transvascular perfusion in this study was 20% v/v. 3) After perfusion of 10% v/v DMSO for 30 minutes, the concentration of DMSO in the muscle tissue of group A1 was stable at about 5% v/v, but the concentration could not meet the vitrification of tissue cells, so the conventional 10% v/v concentration DMSO perfusion is ineffective for deep cryopreservation of large composite tissues; DMSO concentration in muscle tissue of the A2 group is stable at around 12% v/v, which can ensure the vitrification of muscle tissue. 4) The area under the peak of the B1 group and the concentration at 30 minutes in the A1 group and the area under the peak in the B2 group correspond to the concentration in the A2 group at 20 minutes. The area under the peak of the nuclear magnetic resonance spectrum is positive with the target concentration. According to the principle of the ratio, the concentration values of the B and C groups can be calculated in equal proportions. The subcutaneous concentrations of the B1 and B2 groups did not reach 10% v/v. Therefore, it was considered that the simple perfusion method could not achieve the vitrification concentration of the subcutaneous tissue in the large composite tissue. 5) The muscles and blood vessels around the C group were not detected. The DMSO peak is detectable only in the subcutaneous tissue. This might be due to the skin barrier and the thick composite tissue texture. The protective agent cannot

penetrate in a short time, but even the highest concentration of the C1 group is converted to the subcutaneous tissue. Still it did not reach the target concentration of 10% v/v, indicating that the simple immersion method cannot effectively vitrify the bulk composite structure, which explains the reasons for the failure of many simple protective agent soaking experiments.

Conclusions

At present, the formulation and introduction method of large-sized composite tissue cryoprotectant are still inconclusive. This study showed that DMSO cannot effectively vitrify the rabbit hind-limb model by arterial perfusion or whole immersion, and the effective combination of both is needed to solve this problem. The final method of the puzzle requires further research.

References:

1. Jia X, Yu Y, Li D: [Investigation on the effect of trehalose on α -actinin in cryopreserved human skin.] *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi*, 2008; 22(4): 446–49 [in Chinese]
2. Saheli R, Sneha A, Poonam K et al: A simple and serum-free protocol for cryopreservation of human umbilical cord as source of Wharton's jelly mesenchymal stem cells. *Cryobiology*, 2014; 68(3): 467–72
3. Rajagopal K, Chilbule SK, Madhuri V: Viability, proliferation and phenotype maintenance in cryopreserved human iliac apophyseal chondrocytes. *Cell Tissue Bank*, 2014; 15(1): 153–63
4. Forriol F, Longo UG, Alvarez E et al: Scanty integration of osteochondral allografts cryopreserved at low temperatures with dimethyl sulfoxide. *Knee Surg Sports Traumatol Arthrosc*, 2011; 19(7): 1184–91
5. Liu G, Yulin LI, Jian S et al: [Effect of cryopreservation on growth and osteogenesis of human adipose-derived stem cells.] *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi*, 2010; 24(10): 1224–27
6. Xia Z, Duan X, Murray D et al: A method of isolating viable chondrocytes with proliferative capacity from cryopreserved human articular cartilage. *Cell Tissue Bank*, 2013; 14(2): 267–76
7. Zhang JM, Zhang YC, Ruan LH et al: Optimizing cryoprotectant perfusion conditions for intact ovary: A bovine model. *J Assist Reprod Genet*, 2012; 29(11): 1255–60
8. Xiao-wan W: Vitrification of large pieces of human ovarian tissues and xenotransplantation to nude mice after cryopreservation. Thesis for degree of Master of Zhengzhou University (Security level: Published). Zheng Zhou, Henan, China. 2016; R714.8
9. Kim SS: Assessment of long-term endocrine function after transplantation of frozen-thawed human ovarian tissue to the heterotopic site: 10-year longitudinal follow-up study. *J Assist Reprod Genet*, 2012; 29(6): 489–93
10. Leenaars CHC, Freymann J, Jakobs K et al: A systematic search and mapping review of studies on intracerebral microdialysis of amino acids, and systematized review of studies on circadian rhythms. *J Circadian Rhythms*, 2018; 16: 12
11. Dhanani JA, Parker SL, Lipman J et al: Recovery rates of combination antibiotic therapy using *in vitro* microdialysis simulating *in vivo* conditions. *J Pharm Anal*, 2018; 8(6): 407–12
12. An Z-L, Ding K, Liu C-S et al: [Dimethyl sulfoxide acts as a protective agent to perfuse rabbit amputated limbs: The relative recovery of local drug concentrations.] *Chinese Journal of Tissue Engineering Research*, 2015; 19(24): 3855–59 [in Chinese]
13. Zhen Z, Zexing Z, Shuming Z: [Establishment of dimethyl sulfoxide standard curve by freezing point osmometer.] *Lab Med Clinic*, 2015; 12(08): 1101–2 [in Chinese]
14. Rahimi R, Farahmand H: ^1H NMR metabolic profiling of the cryopreserved spermatozoa of the wild endangered Persian sturgeon (*Acipenser persicus*) with the use of beta-cyclodextrin as an external cryoprotectant. *Fish Physiol Biochem*, 2019; 45(3): 1029–40
15. Han X, Ma L, Benson J et al: Measurement of the apparent diffusivity of ethylene glycol in mouse ovaries through rapid MRI and theoretical investigation of cryoprotectant perfusion procedures. *Cryobiology*, 2009; 58(3): 298–302
16. Osei-Bempong C, Ghareeb AE, Lako M et al: Defining the optimal cryoprotectant and concentration for cryopreservation of limbal stem cells. *Cryobiology*, 2018; 84: 98–102
17. Cui X, Gao DY, Fink BF et al: Cryopreservation of composite tissues and transplantation: preliminary studies. *Cryobiology*, 2007; 55(3): 295–304
18. Zeng-tao W, Chunxia W, Lei Z et al: [One case of replantation of 81 fingers in deep hypothermia.] *Shandong Medical Journal*, 2004; 3: 29 [in Chinese]
19. Mouttham L, Fortune JE, Comizzoli P: Damage to fetal bovine ovarian tissue caused by cryoprotectant exposure and vitrification is mitigated during tissue culture. *J Assist Reprod Genet*, 2015; 32(8): 1239–50
20. Mukherjee IN, Song YC, Sambanis A: Cryoprotectant delivery and removal from murine insulinomas at vitrification-relevant concentrations. *Cryobiology*, 2007; 55(1): 10–18
21. Westphal JR, Gerritse R, Braat DDM et al: Complete protection against cryo-damage of cryopreserved whole bovine and human ovaries using DMSO as a cryoprotectant. *J Assist Reprod Genet*, 2017; 34(9): 1217–29
22. Hughes SM, Ferre AL, Yandura SE: Cryopreservation of human mucosal tissues. *PLoS One*, 2018; 13(7): e0200653
23. Davidson AF, Glasscock C, McClanahan DR et al: Toxicity minimized cryoprotectant addition and removal procedures for adherent endothelial cells. *PLoS One*, 2015; 10(11): e0142828
24. Correction to: Cryoprotectant toxicity: Facts, issues, and questions, by best BP. *Rejuvenation Res*, 2015; 18(5): 422–36. *Rejuvenation Res*, 2018; 21(1): 87

Limitation

Although the skin of rabbits is similar to human skin, the large tissue of rabbit skin is still different from human tissue. In the whole soaking group of the experiment, the concentration of DMSO in different parts of rabbit bulk tissue was lower than that of the 20% v/v perfusion group, even under the condition of 50% v/v DMSO. When DMSO reaches 50% perfusion, it is damaging to rabbit tissues. For this reason, we did not conduct further studies on the overall concentration of DMSO in higher concentrations.

Conflict of interest

None.