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Viability of Cells in Cryopreserved Canine Cardiovascular Organs for Transplantation

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Abstract

To determine applicability of the cryopreservation procedure for vessel grafts, the viability of endothelial cells (ECs) among the whole cells in three kinds of organs artery, vein, trachea in mongrel dogs was evaluated on the basis of histological analysis. The *Griffonia simplicifolia agglutins* - fluorescein isothiocyanate (GSA-FITC) and propidium iodide (PI) double staining methods were combined with flow cytometry (FCM), which was able to simultaneously determine the viability of whole cells and ECs from the same tissue, were performed after harvesting, after antibiotic solution treatment, and after cryopreservation and thawing. In most cases, the viability of ECs is lower than that of whole cells from veins and arteries. The viability of whole cells in veins was maintained until the antibiotic solution treatment and then decreased significantly after cryopreservation and thawing, while the ECs began to decrease significantly after the antibiotic solution treatment and more markedly decreased after thawing. The viability of ECs and whole cells from arteries was similar to that of the veins' conditions. The viability of whole cells from the trachea decreased with a similar pattern to that of the ECs from vessels. In consideration of maintaining cell viability among the three kinds of organs, the viability of arteries was better than that of the others. The cells in the trachea demonstrated a lower viability than the vessels. The effect of antibiotic solution treatment on the reduction of cell viability depends on the treatment time and temperature.

Key Words: Viability, endothelial cells, vessels, trachea, cryopreservation

INTRODUCTION

The cryopreservation of tissue at $-70^{\circ}C$ to $-196^{\circ}C$ offers the prospect of indefinite storage. The improvement in the tissue-preservation techniques, such as the introduction of cryoprotectant agents and controlled freezing-rates, have achieved the practical storage of human blood vessels which are ready to use for transplantation.[1]

After transplantation, determining the viability of endothelial cells (ECs) provides very important information about the function and durability of the implanted tissue, because ECs express immune-related functions in monocytes and macrophages, and play an important role in physiologic hemostasis and blood vessel permeability.[2-4] The replacement of the damaged region with transplanted-cryopreserved vessel is done to maintain the functional integrity of the vessel, but the graft acts only as a conduit with severe endothelial damage.[4,5] The failure of transplanted cryopreserved-tissue reflects the cellular or architectural damage of the tissue-capillaries due to the ice formation during the freezing process and to the reperfusion injury. Additionally, the damage to endothelium of the vessel graft that occurs during the implantation has been considered to be a cause of graft thrombosis.[5] Furthermore, the intact endothelium actively produces prostacyclin, antithrombin III, and plasminogen activator. Injury to the ECs results in the liberation of tissue factor procoagulant, and injury to smooth muscle cells evokes modulation and hyperplasia.[6]

Recent studies suggest that certain cells in a given species can exhibit distinct cell surface glycosylation properties differing from the most other cells.[7] For example, in human tissue, it has been reported that the tetrameric, *Griffonia simplicifolia agglutins* (GSA) consisting of five isolectins with two types of subunits

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in different combinations, shows prominent binding only to the α-D-galactosyl residue of vascular cells.[7] FITC has been used as a basic binder to antibodies, agglutins, and immunoglobulins, and can be detected at 515 nm on flow cytometry (FCM).[7,8] PI, which is able to be detected at 615 nm on FCM, intercalates DNA double strands in dead cells without regard to the cell type, as their membrane integrity is lost.[8,9] GS1, an isotype of GSA, has a specific characteristic of binding to EC.[6] Therefore, the staining of FITC conjugated with GS1 detects ECs from other cells, while the double staining of cells by the GS1- FITC and PI can determine the viability of whole cells in a tissue including ECs at once by the FCM.

The arterial-wall is relatively thick and contains greater amounts of smooth muscle and elastic components than the vein-wall. Arterial specimens are easily distinguished from veins, since the thick-wall arteries maintain an open lumen, whereas the thin-walled veins frequently appear more or less collapsed after procurement. The vein-wall consists of three basic tunics, a tunica intima, media and adventitia, but the arterial-wall has additional internal and external elastic laminae. Partly due to this, the boundaries between the three tunics in veins are less distinct than in arteries. The trachea is also an important thoracic organ, in which surface is lined by epithelial cells. The irritation of the epithelium leads to an increase in the number of mucous cells, and a prolonged and intense irritation triggers a local transformation of the epithelium to stratified squamous metaplasia as chronic bronchitis.[10] Tracheal reconstruction following wide resections remains a critical problem. Recent trials to replace the trachea with an arterial tissue are considering the potential of vascularization, and some studies have demonstrate that the replacement of the trachea by cryopreserved aorta is technically feasible and does not evoke immunologic reaction.[10,11]

To prevent microbial contamination, sterilization of the tissue using antibiotics before starting cryopreservation is a critical procedure for clinical use.[11] The cryopreserved vessel allograft demonstrates a rather improved resistance to infection than a fresh allograft, for cryopreservation preserves an amophorous and fibrillar extracellular matrix that is immunologically inert and protects the graft from autolysis. The allograft appears to keep its integrity, which results in a reduced surface thrombogenicity and reduces the risk of hematogenous bacterial colonization.[12] Antibiotic loading, resulting from the 48 hrs decontamination in a medium supplemented with antibiotics performed before cryopreservation of the allograft may have contributed to the increased resistance to infection of aortic allografts observed in animals.[13]

The purpose of this study was to determine the applicability of cryopreservation procedure for vessel grafts. The differential viability determination of ECs from whole cells in three kinds of canine organs-artery, vein, and trachea during cryopreservation was on the basis of histological analysis. Additionally, the effect of antibiotic solution treatment on viability was also examined by varying treatment-times and temperatures of the solution.

MATERIALS AND METHODS

Procurement of cardiovascular organs

Mongrel dogs with 19.4 ± 2.5 kg of body weight were supplied from the laboratory animal center, Yonsei University (Seoul, Korea), four of these were males. Anesthesia was induced by subcutaneous injection of atropine (1 ml) and intravenous injection with ketamine (5 mg/kg). Succinylcholine (0.2 ml) was administered under supplying 100% O_2 inhalation, and anesthesia was maintained by continuous inhalation of enflurane. Following intravenous injection of pancuronium (0.04 mg), three kinds of cardiovascular organs- veins, arteries, and trachea, were procured from six mongrel dogs in order to investigate the viability-variance of vascular and tracheal cells during cryopreservation. The jugular vein, femoral vein, superior vena cava (SVC), inferior vena cava (IVC), carotid artery, femoral artery, internal mammary artery, and trachea were procured from each dog. To investigate the effect of the antibiotic solution treatment, only veins were procured from four dogs. The procured organs were washed twice by phosphate buffered saline (PBS) to discard blood coagulants which may affect ECs viability, and were stored in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) at 4°C.

A guide for the care and use of the laboratory animals was maintained, and the Animal Care Committee of the Yonsei University approved the experimental conditions.[14]

Harvest of cells from organ tissue

For vascular ECs that were to be used for damage caused by mechanical stresses or blood clots,[6] the procured organs were thoroughly washed by PBS without using mechanical devices such as homogenizers or cell-scrappers. The specimen was excised by a surgical blade, and only the inner side of the vessel-walls were placed in an enzyme solution consisting of collagenase 1A (500 unit/ml, Sigma, St. Louis, MO, USA) and trypsin (2.5 g/L, Sigma, St. Louis, MO, USA), which is mixed to 2 : 1 at volume, and incubated at 37°C for 1 hour.[15] After incubation, the enzyme solution was diluted by PBS in order to stop enzymatic reaction, and then the same process was once repeated by replacement with fresh enzyme solution. All the diluted PBS solution was collected and centrifuged at 130×g for 10 min. The resulting precipitate was regarded as the whole cells dissociated from the organ tissue.[16]

Antibiotic solution treatment

The vascular and tracheal specimens were placed in an antibiotic-nutrient solution at 37°C for 4 hrs (n=12). The antibiotic-nutrient solution was composed of cefoxitin (Choongwae Pharma. Co., Kyung-gi do, Korea), lincomycin (Kukje Pharma. Ind. Co. Ltd., Seoul, Korea), vancomycin (Whanin Pharma. Co., Kyung-gi do, Korea) and polymyxin B (Sigma, St. Louis, MO, USA) in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) (Table 1).[17]

To evaluate the effect of antibiotic solution treatment on cell viability of veins before cryopreservation, the cells from the jugular vein, femoral vein, SVC and IVC were treated with the antibiotic solution for 4, 12, 24 hrs at 25°C (n=12).

Cryopreservation and thawing

A conventional cryopreservation method was applied.[17] By inserting a stopcock into the lumen, an extremity of the vessel-specimen was closed and branches were tied. In freezing solution, the tied-specimen was distended at 14.7 mmHg to prevent shrinkage, resulting in a decreasing viability of ECs. After completely filling the lumen with the freezing solution, the opposite extremity was also tied, and placed in the freezing solution. (Table 1) The trachea-specimen was not tied. Finally, the specimen was placed in a commercial freezing bag and frozen at −70°C in a deep-freezer (Ultra-low temperature freezer, MDF-U2086S. Sanyo Electric Co. Ltd., Gunma, Japan), and was transferred to a liquid nitrogen tank (Cryobiological Storage System; CY 50900. Barnstead Thermolyne Co., Dubuque, Iowa, USA) at −196°C for cryopreservation. After 3 weeks, the freezing bag was thawed in water at 70±10°C and the specimen was treated by solution A and B at room temperature until the specimen-temperature reached 37°C. The thawing rate was 2.5°C/min.

GS1-FITC/PI double staining assay on flow cytometry

The precipitate obtained from centrifugation, which is the dissociated cells, was re-suspended by PBS. In a test tube, 0.004% GS1-FITC (ICN Biomedicals Inc. Aurora Ohio, USA) mixed with 2 mM $CaCl_2$ and 2 mM $MgCl_2$ in PBS and 20 μM PI

Table 1. Composition of Antibiotic Solution, Freezing Solution and Thawing Solution Used in Cryopreservation

Antibiotic solution (μg/ml)		Freezing solution (%)		Thawing solution	
				Solution A	Solution B
Cefoxitin	240	RPMI 1640	80	DMEM	DMEM
Lincomycin	120	DMSO	10	Manitol 5M	FCS 10%
Polymixin B	100	FCS	10	FCS	10%
Vancomycin	50				

DMEM is Dulbeccos minimum essential medium (Sigma, St. Louis, MO, USA).
DMSO is dimethyl sulfoxide.
FCS is fetal calf serum.

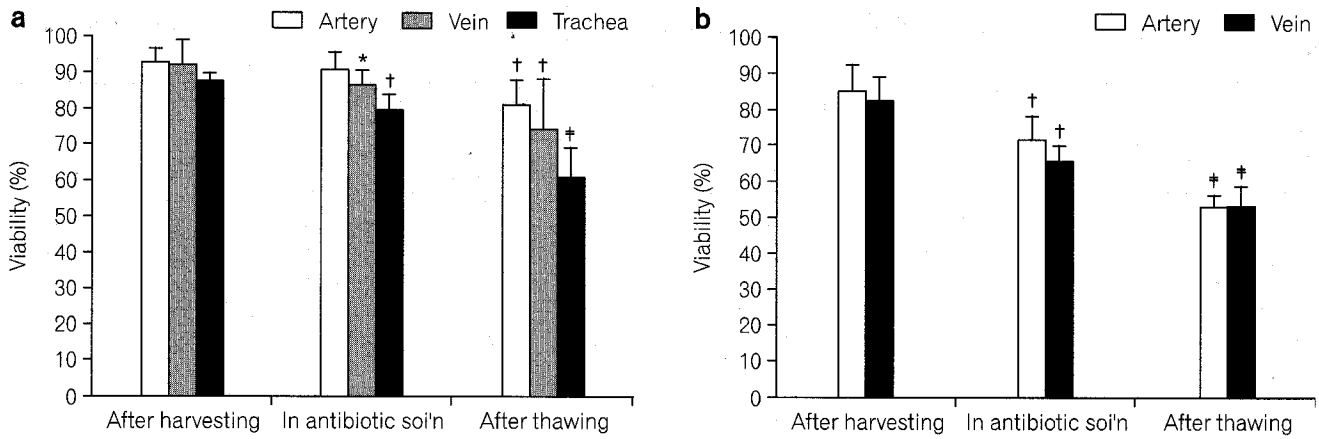

*Fig. 1. Viability of ECs from whole cells in three kinds of organs. (a) Whole cell viability (b) ECs viability (n=12). *p <0.1, †p <0.05, ‡p <0.001: comparison with viability after harvesting as positive control.*

(Sigma, St. Louis, MO, USA) were added to the dissociated cell-suspending PBS solution.[4] The intensities of two fluorescences, GS1-FITC and PI, in the specimen were simultaneously detected in the dotplot and the histogram of FCM (FACSCalibur, Becton and Dickenson, San Jose, USA). For the determination of tracheal cell viability, only the total cell viability was determined by adding the 20 μM PI into the PBS, as the trachea does not include ECs but mainly consists of epithelial cells. The intensities of PI in the specimen were detected by the FCM histogram. The viabilities of ECs and whole cells were compared with one another by statistical analysis using student T-test.

RESULTS

Detected viability of ECs from whole cells in three organs

With regard to viability after harvesting as a positive control, viability after the antibiotic solution treatment for cryopreservation and after thawing was compared in each organ (Fig. 1).

In general, the viability of ECs was lower than that of whole cells in vessels. The viability of whole cells in veins decreased slightly ($p<0.1$) after antibiotic solution treatment but decreased significantly ($p<0.05$) after cryopreservation and thawing, while the viability of ECs decreased significantly ($p<0.05$) following treatment with antibiotic solution and more steeply ($p<0.001$) after thawing. The viability of ECs from arteries showed similar pattern to that of veins. The antibiotic solution treatment did not effect the viability of the whole cells in the arteries, but there was a significant decrease in viability ($p<0.05$) after cryopreservation and thawing. The viability of whole cells dissociated from trachea decreased with a similar pattern to the viability of ECs from veins or arteries; it decreased significantly ($p<0.05$) after antibiotic solution treatment but more apparently after thawing ($p<0.001$).

As the viability of arteries was better than that of the others, viability of arteries was regarded as positive for the other two organs (Table 2). There was no significant difference in cell viability between arteries and veins, but a slight difference ($P<0.1$) was observed following antibiotic solution treatment. The ECs were also different ($P<0.1$) after treatment with antibiotic solution. In comparison with the arteries, the viability of the trachea, however, began to decrease significantly ($p<0.05$) following antibiotic solution treatment, and decreased continuously after cryopreservation and thawing ($p<0.05$). In consideration of maintaining cell viability among the three kinds of organs, the viability of arteries was better than that of the others, and that of the trachea was less than both arteries and veins.

Cell viability affected by the condition of antibiotic solution treatment in cryopreservation

The effects of time and temperature variances of

Table 2. Statistical Comparison of Cell Viability among Arteries, Veins and Tracheas. They Were the Same Value with Fig. 1.

Canine organ (n=12)	Whole cell viability (%)			ECs viability (%)	
	Artery	Vein	Trachea	Artery	Vein
After harvesting	92.7±3.8	91.9±6.8	87.1±2.6	84.9±6.4	82.3±7.5
In antibiotic solution	90.4±4.7	86.3±4.1*	79.1±4.3†	71.5±4.4	65.6±6.6*
After thawing	80.5±7.4	73.7±14.1	60.7±7.9†	53.1±5.4	53.6±3.4

* $p<0.1$, † $p<0.05$, † $p<0.001$: comparison with the cell viability of arteries as positive control.

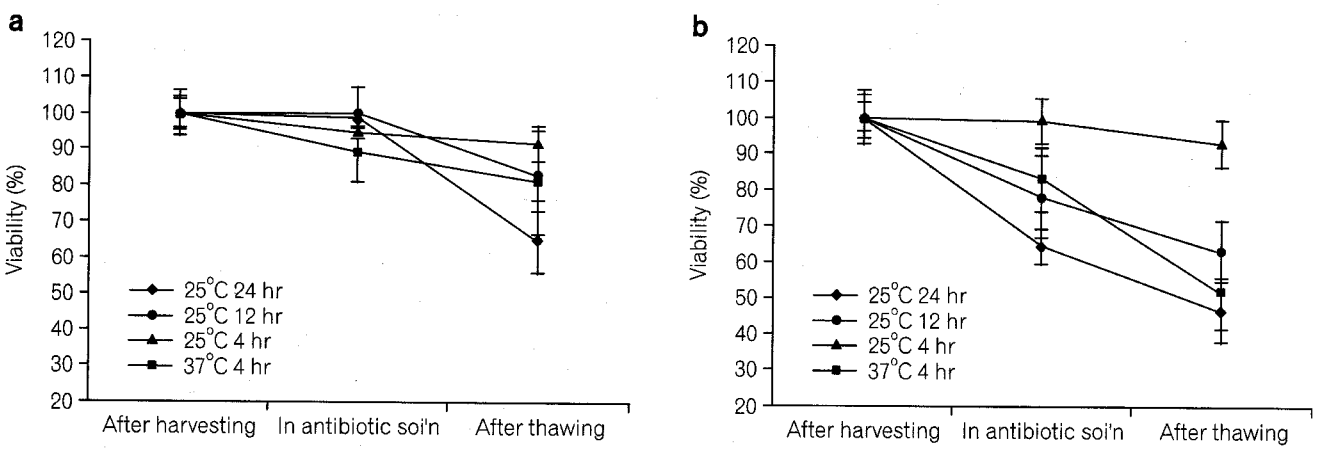

Fig. 2. Venous Cell viability in various conditions of antibiotic solution treatment for cryopreservation. The veins were femoral veins, jugular veins, IVC and SVC (n=12). The symbol indicates the temperature and time of antibiotic solution treatment. (a) whole cell viability (b) ECs viability.

antibiotic solution treatment on the viability of cells following cryopreservation and thawing were investigated. The viability of whole cells in each group decreased differently following antibiotic treatment, and was dependent on the time and temperature of treatment. At 25°C, the viability of whole cells decreased more apparently in proportion to the increasing time of the antibiotic solution treatment. Thus, the viability of cells treated for 24 hrs decreased the most significantly, while the viability after four hours of treatment decreased the least. As the antibiotic solution temperature increased, the venous cellular viability decreased. Referring to the four hours of treatment time, the specimens treated at 25°C demonstrated higher viability than those treated at 37°C (Fig. 2a). Meanwhile, in ECs, viability decreased with a similar to that of the whole cells, as it apparently began to decrease following the antibiotic solution treatment. This also was dependent on the time of antibiotic solution treatment at 25°C with a similar pattern to that of the whole cells, but the viability decreased more significantly than that of the whole cells. Following antibiotic treatment for 4 hours, there was no significant difference between the groups treated at 25°C and 37°C, whereas the group treated at 37°C decreased more significantly after cryopreservation and thawing than that treated at 25°C (Fig. 2b).

DISCUSSION

The endothelial cells play important roles in functions such as resistance to thrombosis, maintenance of hemostasis and modulation of vascular smooth muscle activity in vascular graft, and the maintenance of cellular viability during preservation of the vascular graft is highly desired.[18] Therefore, detecting the viability of the endothelium is necessary in order to distinguish it from that of whole cells in

donor vessel.[18] GS1, which has a specific characteristic of attaching to the endothelial cell membrane, was conjugated by FITC.[19] These GS1-FITC stained ECs were easily distinguished from the whole cells, and PI stained cells, either ECs or whole cells, indicates death of cells through histogram of FCM. On this basis, non-viable ECs, which are stained by both GS1-FITC and PI, could be distinguished from the viable ECs, which are stained only by GS1-FITC. Other cells, which are not stained by GS1-FITC, were simply distinguished by PI staining as positive for all non-viable cells. The number of viable ECs out of total number of tissue cells was calculated by the dotplot of FCM. In most cases, the viability of the ECs was less than that of whole cells in the arteries and veins, and the ECs were assumed to be more sensitive to external environments than other structural cells. Therefore, the viability of ECs was considered to be a more effective indicator of susceptibility in each cryopreservation procedure.

Among the thoracic organs, viability of tracheal cells was significantly less than the other organs, for the trachea is mainly composed of epithelial cells and no endothelial cells.[10] The use of homograft, either fresh or preserved, has a very frequent tendency toward necrosis and/or stenosis, and in practice requires immunosuppression,[20,21] although a recent experimental study using a long-term cryopreserved tracheal allograft demonstrated an improved result.[22] Some experimental approaches using a segment of cryopreserved aorta aimed at replacing the trachea by a quite simple surgical procedure also reported no immune reactions.[23,24]

In this study, there was no significant difference observed between veins and arteries, but the viability of both whole cells and ECs in arteries was more maintained than that of veins following antibiotic solution treatment. There was no significant difference in the ECs out of whole cells between arteries ($32.35 \pm 11.1\%$) and veins ($34.65 \pm 10.1\%$). This suggested that the minute difference of viability between the two tissues may result not only from cell composition but also from other factors. In general, the veins accompany the corresponding arteries as a rule but have a larger diameter. Moreover, as the tunica media in veins is much thinner than in arteries, veins have thinner walls than arteries of the same order of magnitude, and this should be related to the much lower venous blood pressure. Finally, the vein wall contains more connective tissue than in arteries, although the veins in vertebrates including humans and other animals are relatively rich in elastic tissue and accordingly are quite distendable. Due to the thinner wall, veins frequently are collapsed in tissue section.[10] On the other hand, the findings imply that vascular muscle damage by cryopreservation may protect the arterial graft from immune reaction and graft disease.[25]

It has been reported that an important matter in cryopreservation occurs when the cells transpass through the critical freeze-thaw equilibrium temperature range ($-10^{\circ}C$ to $-40^{\circ}C$), and cellular damage is caused by intracellular ice formation at these temperature.[7] The dose and composition of antibiotic solution might also largely affect the damage of cells. In terms of impacts on cell viability by pretreatment with antibiotic solution, it was considered that the decisive factor decreasing the viability of endothelial cells as well as whole cells might be the duration of retention in an antibiotic solution in the short-term cryopreservation of cardiovascular organs.[26]

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