—Originals—

Effects of temperature and preservation time on the pharmacological response of isolated vascular endothelial and smooth muscle function

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Abstract

In clinical transplantation and cardiovascular surgery, cold preservation is usually used because it is a simple method. However, the established temperature is by no means exact. The aim of this study was to find the optimum storage temperature for preservation of the vasculature by observing the pharmacological endothelium and smooth muscle response. The thoracic aorta of 36 male Wister rats were studied in organ baths：as fresh control, after 24 hours, 48 hours and 72 hours of storage at 0.5℃, 4℃ and 8℃ in Krebs-Henseleit bicarbonate (KHB) solution. Acetylcholine (Ach) was used to elicit endothelium-dependent relaxation, and sodium nitroprusside (SNP) to elicit smooth muscle-dependent relaxation. The contractility caused by Phenylephrine (Ph) was influenced by time but before 48 hours it was not influenced by preservation temperature. Significant responsive deterioration by Ach and SNP was seen after 24 hours of storage at 0.5℃ as compared with storage at 4℃ and 8℃. The endothelium-dependent relaxing function and smooth muscle-dependent relaxing function were best preserved at 4℃ and 8℃. These results indicate that precise temperature control is necessary for vessel preservation in clinical situations. (J Nippon Med Sch 1999；66：15—20)

Key words：cold preservation, temperature, time, endothelium, smooth muscle

Introduction

Recently transplant surgery has been performed all over the world, which has made it possible to provide medical treatment for previously incurable diseases. One of the interesting issues regarding transplant surgery is how to preserve extracted tissues and organs for an extended period. Clinically, transplantation tissue and organ "freshness" has a strong influence on survival and function. Therefore, many studies have fried to fiat to maintain freshness. In clinical transplantation and cardiovascular surgery, cold preservation is usually used because of its simplicity. In cold preservation, the preservation temperature is commonly established at 4℃, but this established temperature is by no means exact. The aim of this study was to find the optimum storage temperature for preservation of the vasculature by observing pharmacological endothelium and smooth muscle response.

Materials and Methods

The animals were treated in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health (NIH publication 85-23, revised 1985). After obtaining the approval of the Animal Experimental Ethical Review

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Committee, Nippon Medical School, 36 male Wister rats (body weight 196±8 g ; mean±SD) were used. The rats were anesthetized with pentobarbital (70 mg /kg, i.p.), intubated and mechanically ventilated with oxygen. Heparin sodium, 10 mg/kg, was given intravenously before the thoracotomy. The thoracic aorta was removed immediately after the thoracotomy, from the diaphragm to the aortic arch (about 3 cm). Then the aorta was placed in oxygenated modified Krebs-Henseleit bicarbonate (KHB) solution (composition (mM) : KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, $CaCl_2$ 2.54, NaCl 119, $NaHCO_3$ 25 and glucose 11 ; pH was adjusted to 7.4). The aorta was dissected free of fat and connective tissue and care was taken not to damage the endothelial cell layers or stretch the vessels. The vessels were separated, and 12 vessels placed into each of the 0.5℃, 4℃ and 8℃ preservation groups. After being washed into the lumen by oxygenated KHB solution, each dissected vessel was cut into 3mm width aortic rings. PCR thermal cycler MP TP-3000 (Takara Shuzo Co.) for DNA synthesis was used to maintain the same preservative temperature continuously. Soon after dissection (control), after 24 hours, 48 hours and 72 hours preservation periods, measurement of pharmacological contraction and relaxation were performed by the method described below.

(1) Measurement of contractility and relaxation

Using an isometric tension measurement system (Easymagunus, UFER Co.), we mounted each aortic ring between 2 stainless wires and fixedit. The other was attached to a transducer (TB-611T, Nihon Kohden) connected to a computer (MacLab/s, ADInstruments) to change the electrical signal into contracting and relaxing tensions. The data were stoved on a computer (MacLab Chart/s, Macintosh 7200). The solution bubbled continuously in a mixture of 95 %O_2 and 5% CO_2, and was maintained at 37℃. The tissue was equilibrated for 2 hours under a resting tension of 2 grams by changing the bath fluids every 15 min. After a 2 hour equilibration period, 3×10^{-7}M phenylephrine (Ph), 10^{-5}M acetylcholine (Ach) and 10^{-7}M sodium nitroprusside (SNP) were added to the bath in this order, and the isometric tension was measured and recorded. Ach and SNP were added when the maximum effect had been produced by the proceeding drug.

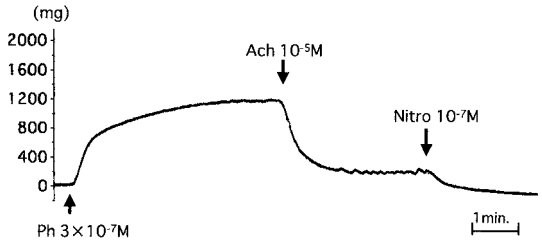

Fig. 1 Acutal recording of vascular contractility and relaxation. (Ph=phenylephrine : Ach=acetylcholine : Nitro=sodium nitroprusside)

(2) Data analysis

All data were expressed as the mean ± the standard deviation of the mean (S.D.). Contracting tension with 3×10^{-7}M Ph was expressed as P. The next relaxing tension with Ach was A. The next relaxing tension with SNP was N. (P-A)/P represented the endothelial responsive ratio. (P-N)/P represented the smooth muscle responsive ratio. (P-A)(P-N)/P×P represented the vascular responsive ratio. Statistical evaluation in each group on P, (P-A)/P, (P-N)/P and (P-A)(P-N)/P×P at 0 hour (control), 24 hours, 48 hours and 72 hours was performed with one-way repeated measures ANOVA. Statistical evaluation between groups regarding P, (P-A)/P, (P-N)/P and (P-A)(P-N)/P×P at 0 hours (control), 24 hours, 48 hours and 72 hours was performed with one-way factorial ANOVA using Bonferroni's test to perform the multiple comparisons. A p value of less than 0.05 was considered statistically significant.

Results

In the case of normal vessels, 3×10^{-7}M of Ph gives a submaximal tone (about 80% of maximum) as previously determined in our laboratory. This concentration of Ph was used to obtain the initial contractility. After the initial contractility was obtained, Ach (10^{-5} M) was added to the bath. This concentration of Ach gives a relaxation of more than 90%. In the same way, SNP (10^{-7}M) gives about 100% relaxation. Vascular endothelial damage was investigated by measuring the changes in vasodilation as a result of endothelium-derived relaxing factor release by Ach after the initial contractility was obtained by Ph (**Fig. 1**). The changes with time at 0 hours (control), 24 hours, 48 hours and 72 hours of maximal vascular contractility after the addition of 3×10^{-7}M Ph in the 0.5℃, 4℃ and 8℃ preservation groups are shown in **Table 1** and **Fig. 2**.

Table 1 Vascular tension (mg) with 3×10^{-7} M phenylephrine

Group	0h (control)	24h	48h	72h
0.5℃	1,356 ± 180	949 ± 117	1,086 ± 195	892 ± 215 *
4℃	1,241 ± 178	1,089 ± 232	973 ± 130	1,106 ± 118
8℃	1,335 ± 157	1,072 ± 149	1,151 ± 182	1,140 ± 227

All data are expressed as the mean ± the standard deviation of the mean (n = 12 rats in each group). * $p < 0.05$; 0.5℃ vs 4℃ and 8℃

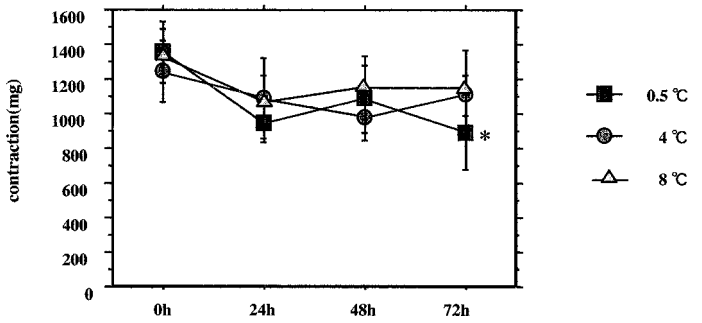

Fig. 2 Maximal vascular contractility (P) with 3×10^{-7}phenylephrine. Each point shows the mean ± the standard deviation of the mean (n=12 rats in each group). *$p < 0.05$; 0.5℃ vs 4℃ and 8℃

Table 2 Minimal vascular tension (mg) with 10^{-5} M acethylcholine

Group	0h (control)	24h	48h	72h
0.5℃	64 ± 21	199 ± 54 *	710 ± 388 *	553 ± 232 *
4℃	53 ± 19	84 ± 60	84 ± 41	163 ± 84
8℃	79 ± 29	87 ± 42	99 ± 48	250 ± 233

All data are expressed as the mean ± the standard deviation of the mean (n = 12 rats in each group). * $p < 0.05$; 0.5℃ vs 4℃ and 8℃

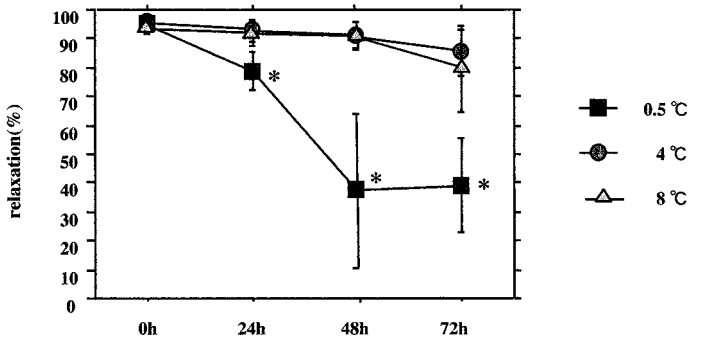

Fig. 3 Endothelial responsive ratio with 10^{-5}M acethylcholine. Each point shows the mean ± the standard deviation of the mean (n=12 rats in each group). *$p<0.05$; 0.5℃ vs 4℃ and 8℃

Progressive changes in each group were found to be significant (0.5℃ : $p<0.0001$; 4℃ : $p=0.0017$; 8℃ : $p=0.0074$). When comparing each group at each time, 0.5℃ and 4℃ preservation groups at 72 hours were found to be significantly different ($p=0.0358$ and $p=0.013$). The changes with time of minimal vascular tension and endothelial responsive ratio, (P-A)/P, after relaxation with 10^{-5}M Ach are shown in **Table 2** and **Fig. 3**. The progressive changes of each group were found to be significant (0.5℃ : $p<0.0001$; 4℃ : $p=0.0002$; 8℃ : $p=0.0011$). When comparing each group at each time, the 0.5℃ and 4℃ preservation groups at 24 hours, 48 hours and 72 hours ($p<0.0001$), the 0.5℃ and 8℃ preservation groups at 24 hours, 48 hours and 72 hours ($p<0.0001$) were found to be significantly different. The changes with time of minimal

Table 3 Minimal vascular tension (mg) with 10^{-7} M sodium nitroprusside

Group	0h (control)	24h	48h	72h
0.5℃	16 ± 12	32 ± 36*	88 ± 63*	117 ± 69*
4℃	18 ± 13	10 ± 9	11 ± 11	8 ± 12
8℃	16 ± 12	9 ± 12	4 ± 7	6 ± 10

All data are expressed as the mean ± the standard deviation of the mean (n = 12 rats in each group). * $p < 0.05$; 0.5℃ vs 4℃ and 8℃

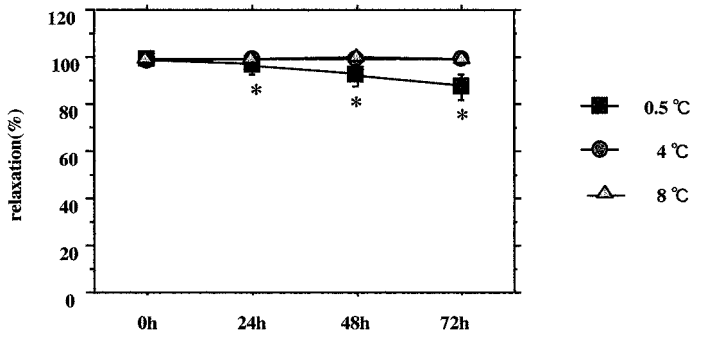

Fig. 4 Smooth muscle responsive ratio with 10^{-7}M sodium nitroprusside. Each point shows the mean ± the standard deviation of the mean (n=12 rats in each group). When error bars are not visible they are hidden by the symbols. *p<0.05 ; 0.5℃ vs 4℃ and 8℃

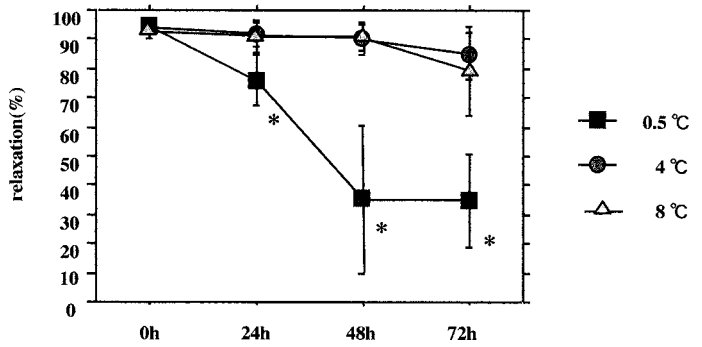

Fig. 5 Vascular responsive ratio. Each point shows the mean ± the standard deviation of the mean (n=12 rats in each group). *p<0.05 ; 0.5℃ vs 4℃ and 8℃

vascular tension and the smooth muscle responsive ratio, (P-N) /P, after relaxation with 10^{-7}M SNP are shown in **Table 3** and **Fig. 4**.The progressive changes of the 4℃ and 8℃ preservation groups were not found to be significant but those of the 0.5℃ preservation group found to be significant ($p<0.0001$). When comparing each group at each time, the 0.5℃ and 4℃ preservation groups at 24 hours ($p=0.0143$), 48 hours and 72 hours ($p<0.0001$), and the 0.5℃ and 8℃ preservation groups at 24 hours ($p=0.0435$), 48 hours and 72 hours ($p<0.0001$) were found to be significantly different. The changes with time of the vascular responsive ratio, (P-A) (P-N)/P × P, are shown in **Fig. 5**. The progressive changes of each group were found to be significant (0.5℃ : $p<0.0001$; 4℃ : $p=0.0009$; 8℃ : $p=0.0024$). When comparing each group at each time, the 0.5℃ and 4℃ preservation groups at 24 hours, 48 hours and 72 hours ($p<0.0001$), and the 0.5℃ and 8℃ preservation groups at 24 hours, 48 hours and 72 hours ($p<0.0001$) were found to be significantly different.

Discussion

The increased understanding of vascular endothelium function since 1980, when Furchgott and Zawadski observed that acetylcholine elicited a vascular relaxation, has been marked[1]. Further research into this field has demonstrated a number of endogenous substances[2]. At first, several substances were believed to

be endothelium-derived relaxing factors (EDRF). One of them has been identified as a very simple molecule, namely, nitric oxide (NO)[3]. The physiological precursor of NO is the amino acid L-arginine, which releases NO in its enzymatic conversion to L-citrulline. NO stimulates guanylate cyclase, thereby resulting in increased levels of cyclic 3', 5' − guanosine monophosphate, which relaxes the vascular smooth muscles[4]. NO has also been shown to inhibit the aggregating and the adhesion of platelets to endothelial cells[5]. If we use vessels as a vascular graft, patency is an important problem and occlusion is a matter of life and death for the grafted organ. As mentioned above, NO influences the viability of vessels. It is known that exposure to low temperatures can impair the basal and stimulated release of EDRF[6]. It is also reported that structural changes are induced by hypothermia using cultured human endothelial cells[7]. The aim of the present study was to investigate the condition of preserved vessels by the pharmacological reaction which depends upon EDRF release (i.e., acetylcholine) and acts directly on the smooth muscles (i.e., nitroprusside)[8, 9].

In this study, the changes in vascular contraction, endothelium-dependent and -independent vasodilation were evaluated according to the methods of the previous studies[10, 11] with some modifications. The previous studies showed that removal of the endothelium completely inhibited the response to acetylcholine, whereas the response to sodium nitroprusside remained unaltered[10, 12]. In the preliminary study, the response of the endothelium intact vascular ring after the addition of SNP did not change whether Ach was added or omitted. And the endothelium denuded vascular ring was not relaxed by Ach, but was relaxed more than 90% with 1×10^{-7}M SNP administration after being precontracted with 3×10^{-7}M Ph. Therefore the progressive responsive deterioration for Ach was thought to be caused by endothelial injury, and the change of response for an activator of vascular smooth muscle soluble guahylate cyclase (SNP) was supposed to be caused by smooth muscle injury during cold preservation.

In the present study the changes with time of maximal vascular contractility after addition of 3×10^{-7}M Ph in the 0.5℃, 4℃ and 8℃ preservation groups were found to be significant. The mechanism of contractility after the addition of Ph is constriction by receptor-mediated intracellular Ca^{2+}release. This result indicated that the vasocontractility by Ph was influenced by time, but before 48 hours it was not influenced by the preservative temperature. In this study, damage to vessels when the vessels were harvested was disregarded because all vessels at 0 hours (control) have relaxation of more than 50%[10]. The progressive responsive deterioration for Ach was due to endothelial injury. Severe endothelial injury of the 0.5℃ preservation group was recognized after a preservation period of 24 hours as compared with endothelial of the 4℃ and 8℃ preservation groups. At 48 hours and over, the relaxation when Ach was added was less than 50 %. Smooth muscle injury as a direct pharmacological response to SNP was recognized after the preservation period of 24 hours. In the 4℃ and 8℃ preservation groups, no obvious progressive changes due to the addition of SNP at any time were recognized. In other words, the smooth muscle could be maintained in good condition for 72 hours at 4℃ or 8℃. As mentioned above, the endothelium was more liable to be influenced by the time factor and preservative temperature than the smooth muscle. Severe deterioration of the 0.5℃ preservation group was also recognized in the vascular responsive ratio after 24 hours preservation. From our results, we thought that preservation at 4℃ and 8℃ was better than at 0.5℃. Obvious differences between preservations at 4℃ and 8 ℃ were not acknowledged.

There have been few reports concerning the optimal storage temperature for long-term preservation of the vasculature. Ingemansson et al. reported that EDRF function was best preserved at 4℃ and 8℃, and preservation of vascular smooth muscle function was best at 0.5℃[13]. In the present study, both EDRF function and vascular smooth muscle function were better preserved at 4℃ and 8℃. Although the reason for the difference between our results and those of Ingemansson is unknown, it may be due to the different method of controlling the tissue temperature, different time periods to measure the vascular function, or a different method of measuring vascular function. One of the most important issues for the experiment is how to keep the temperature precise during storage. Ingemansson et al. used refrigerators and put ice-slush in the solution to control the tissue temperature. In contrast, we used a more precise thermal controller. In clinical situations, the maintenance of tissue

temperature at low temperatures is very difficult. As the temperature gets closer to 0℃, the risk of freezing the tissue increases. In the present study, it is impossible to deny tissue injury by freezing despite the use of a precision temperature control machine. In clinical transplantation and cardiovascular surgery, ice-slush is usually used for organ and tissue preservation. The temperature in ice-slush solutions lies between 0℃ and 1.0℃ and easily decreases to near freezing. According to our study, vascular functions were best preserved at 4℃ and 8℃. If the preservative temperature is 4℃ or 8℃ and vessel freezing is avoided, improved quality of vascular preservation could be obtained.

We concluded that 4℃ and 8℃ were better preservative temperatures for long-term preservation than 0.5℃, and emphasized the necessity of precise temperature control to prevent freezing of the vessels in clinical situations.

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