

Functional, Metabolic, and Histological Changes of Vascular Tissues after Warm Ischemia

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We examined functional, metabolic, and histological changes in the aortic tissue of rats after the period of warm ischemia ranging from 0 to 24 hours to determine the window of time in which grafts can be optimally viable for harvest. Sixty aortas from Brown Norway rats obtained after warm ischemia were used and changes in contraction, endothelial-dependent or -independent vasodilatation, cell viability, and histology were examined. Maximal contraction induced by norepinephrine and potassium chloride decreased time-dependently after exposure to warm ischemia. The warm ischemic period when 50% of the maximal contractile response of freshly isolated arteries was preserved, ranged from 6 to 8 hours. Maximal endothelium-dependent relaxation induced by acetylcholine decreased along with the time of warm ischemia. Endothelium-independent relaxation induced by sodium nitroprusside and forskolin was unaltered for up to 9 hours. Cell viability gradually decreased, and a significant negative correlation was found between the warm ischemic period (T : hours) and cell viability (V : %) ($V = 101.9 - 2.35T$; $r^2 = 0.96$; $p < 0.0001$). Cell viability was greater than 70% within 12 hours postmortem. Histologically, after a 9-hour-warm ischemia period, irreversible changes were detected. Results suggest that a period of warm ischemia for up to 6 hours would be acceptable for preservation of tissue viability. (Ann Thorac Cardiovasc Surg 2001; 7: 143–9)

Key words: warm ischemia, ischemic time, vascular function, cell viability

Introduction

Allografts are often used for reconstructive cardiovascular surgery. Preservation of graft viability is essential to the maintenance of graft structure and consequent function. The duration of warm ischemia from donor death to graft harvest is a critical determinant of graft viability.¹⁻³⁾ However, the acceptable warm ischemic period is

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controversial. For cardiac valves, the time from donor death to graft harvest varies from 2 to 72 hours.¹⁻⁹⁾ Some investigators^{2-4,10)} suggested that the use of valves harvested more than 24 hours after donor death is unwise; by then, the valve leaflet cells are nonviable. Only limited data are available, however, concerning the effects after time exposed to warm ischemia on vascular grafts.^{11,12)} To ensure a stable clinical supply of allografts, the time limit of warm ischemia must be clarified. Therefore, in the present experiments, we designed an experimental model to assess the effects of varying warm ischemic time on vasomotor properties, metabolic function, and histology of the vascular tissue.

Materials and Methods

Animals

Male Brown Norway rats weighing 180-250 g were ob-

tained from Seac Yoshitomi Ltd. (Fukuoka, Japan) and were maintained at the Center for Animal Resources and Development, Kumamoto University (Kumamoto, Japan). All experiments adhered to the National Research Council guidelines for the care and use of laboratory animals.

Tissue preparation

The rats were sacrificed by intraperitoneal injections of sodium pentobarbital (50-80 mg/kg). Segments of the thoracic aorta between the aortic arch and diaphragm were excised from rats at six different postmortem intervals (warm ischemic period): immediately after death (H0, n=10) and after maintaining sacrificed rats at room temperature (20-25°C) for 3 (H3, n=10), 6 (H6, n=10), 9 (H9, n=10), 12 (H12, n=10), and 24 hours (H24, n=10). Rectal temperature was measured at the time of sacrifice and when the aortas were excised.

Each excised aorta was placed in Krebs-Henseleit solution comprised of the following (in mM): NaCl 121, KCl 4.9, CaCl₂ 2.6, MgSO₄ 1, KH₂PO₄ 1, NaHCO₃ 20, and glucose 10. The solution was aerated with 95% O₂ and 5% CO₂, and the pH at 37°C was 7.4. The preparation was carefully cleaned of the adherent fat and connective tissue. Intraluminal blood was removed by gentle lavage in the Krebs-Henseleit solution. Care was taken to ensure that the endothelial layer was not damaged.

Isometric tension

For isometric tension studies, aortic segments (10 segments for each postmortem ischemia interval) were cut into rings 5 mm in length. Each ring was suspended between two stainless steel wire hooks. One wire was attached to a fixed tissue support in an isolated tissue bath containing Krebs-Henseleit solution maintained at 37°C and continuously aerated with 95% O₂ and 5% CO₂. The other wire was connected by a silk thread to the force transducer, (Nihon Kohden TB-611T, Tokyo, Japan) through which changes in isometric force were recorded and displayed (Sekonic SS-250F, Tokyo, Japan). The resting tension of 2 g was applied to the ring allowing to equilibrate in Krebs-Henseleit solution for 90 to 120 minutes before testing. The solution was changed every 20 to 30 minutes during the equilibration period. After equilibration, cumulative concentration-response curves to norepinephrine (NE, 10⁻¹⁰ to 10⁻⁵ M) and potassium chloride (10 to 100 mM) were obtained for each preparation by adding the agents, and then, preparations were washed with the bathing solution and equilibrated at least

for 60 minutes before addition of the next agent. From tissues pre-contracted by NE (10⁻⁶ M), cumulative concentration-response curves to acetylcholine (ACh, 10⁻¹⁰ to 10⁻⁴ M), sodium nitroprusside (SNP, 10⁻¹⁰ to 10⁻⁴ M), and forskolin (10⁻¹⁰ to 10⁻⁴ M) were obtained. After the maximal effect of each vasodilating agent was achieved, papaverine (100 μM) was added to determine the maximum relaxation.

Contraction was expressed as grams of isometric force exhibited by the arterial rings in response to contractile agonists. Relaxation elicited by the papaverine was determined as 100% relaxation. Endothelium-dependent and -independent relaxation was expressed as percentages of the maximal relaxation achieved with papaverine. The maximum effect (E_{max}) was defined as the greatest response induced by the agonist. Effective concentrations of the agonist causing half-maximal response (EC₅₀) were determined from each curve by a logistic curve-fitting equation and were expressed as pD₂ values, the negative logarithm of EC₅₀. Drug concentration was expressed as the final molar concentration in the tissue bath.

Norepinephrine hydrochloride, acetylcholine chloride, sodium nitroprusside, and forskolin were all purchased from Sigma Chemical Co. (St. Louis, MO). Papaverine and potassium chloride were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Drugs were dissolved in distilled water, except forskolin, for which a stock solution at 100 μmol/L was prepared in DMSO. Stock solution of NE was prepared in the presence of 2 mg/mL ascorbic acid to prevent oxidation. Drugs were kept at -20°C and were freshly dissolved in distilled water to the appropriate final concentrations.

Calorimetric assay for cell viability

Cell viability in all aortic segments (n=10 per ischemia period) was evaluated based on the enzymatic activity of tetrazolium salts. Smooth muscle cells were harvested with collagenase treatment. The endothelial layer was removed from the aorta by gentle rubbing with a glass rod. Smooth muscle cells were detached from pieces of the rat aorta (20 mm in length) by a 60-minute incubation at 37°C in 0.02% collagenase (200 units/mL) (Amano Pharmaceutical Co., Nagoya, Japan) in Tyrode-HEPES solution with 0.1% papain (Wako) in 15-mL centrifugal tubes. The Tyrode-HEPES solution was comprised of (in mM): NaCl 137, KCl 2.7, CaCl₂ 0.18, MgCl₂ 1.0, glucose 5.6, and HEPES 4.2; its pH was adjusted to 7.4 with 1N NaOH. The cells were resuspended in culture medium TC-199 (Life Technologies Inc., Grand Is-

Table 1. Responses to contractile agents in control rat aorta (H0) and 3 hours (H3), 6 hours (H6), 9 hours (H9), 12 hours (H12), and 24 hours

		H0	H3	H6	H9	H12	H24
Norepinephrine	pD ₂	7.67 ± 0.27	7.48 ± 0.14	7.55 ± 0.27	7.83 ± 0.11	7.60 ± 0.21	7.94 ± 0.57
	E _{max} (g)	2.18 ± 0.40	1.99 ± 0.34	1.28 ± 0.22*	0.44 ± 0.20*	0.26 ± 0.17*	0.11 ± 0.07*
Potassium chloride	pD ₂	1.60 ± 0.04	1.61 ± 0.04	1.78 ± 0.04	1.89 ± 0.12	1.76 ± 0.28	1.60 ± 0.12
	E _{max} (g)	2.99 ± 0.34	2.70 ± 0.30	2.45 ± 0.21**	0.98 ± 0.11*	0.38 ± 0.19*	0.05 ± 0.03*

pD₂: negative logarithm of the molar concentration producing 50% of maximal response. E_{max}: maximal contraction expressed as gram isometric force developed by the ring. Data were given as means ± SD. **p*<0.0001 vs. H0; ***p*<0.001 vs. H0.

land, NY) with 10% fetal calf serum and incubated at 37°C in 5% carbon dioxide. After 1 hour of incubation, freshly prepared WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt; Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan) was added to a final WST-8 concentration, of 0.5 mM, and cells were incubated at 37°C in 5% carbon dioxide for 2 hours. The supernatant from tetrazolium assay was transferred to 96-well microtiter plates (Nunc, Roskilde, Denmark). Optical density for the tetrazolium-produced formazan was determined with an E_{max}TM precision microplate reader (Molecular Devices Co., Sunnyvale, CA) at 450 nm and a reference wavelength of 650 nm. All optical density values were standardized according to the tissue weight and expressed as percentage of the control (H0) group value.

Histological studies

Formalin-fixed segments of the aorta from all six tissue groups (n=10 per ischemic period) were embedded in paraffin, completely sectioned into 5- μ m thick sections, and stained with both hematoxylin and eosin and Elastica-van Gieson. Smooth muscle and endothelial cells were counted and then estimated per 0.1 mm². The numbers of cells were expressed as a percent of the number of cells from the control (H0) tissue.

Statistics

Data are shown as mean ± SD. Statistical analysis was performed with Student's t-test for unpaired data or one-way analysis of variance for multiple comparisons and Bonferroni correction. Correlation between the warm ischemic period and percent of cell viability as determined by WST-8 was assessed by Pearson's correlation coefficient and linear regression analysis. A *p*-value of less than 0.05 was considered statistically significant. Regression lines for cumulative concentration-response curves

and the relation between the warm ischemic period and E_{max} in response to NE or potassium chloride were obtained with a computerized graphics package (DeltaGraph, DeltaPoint Inc., Monterey, CA).

Results

Rectal temperature

No statistical difference in the rectal temperature at sacrifice was found between tissue groups (mean: 36.4±0.3°C). The rectal temperature at 3, 6, 9, 12, and 24 hours after death was significantly decreased (27.1±1.9°C, 24.3±2.1°C, 21.8±1.1°C, 20.5±1.1°C, and 20.7±0.8°C, respectively).

Changes in isometric tension of the preparation after exposure to warm ischemia

Sensitivities (pD₂) to NE and potassium chloride were similar between groups (Table 1). However, E_{max} gradually decreased along with the warm ischemic period in a logarithmic fashion as expressed by a sigmoidal curve-fitting equation (Figs. 1 and 2). Maximal contraction induced by both agents was unaltered for up to 3 hours after warm ischemia. More than 50% of the E_{max} was preserved in the control (H0) group at 6.17 hours for NE and at 7.83 hours for potassium chloride.

Sensitivity and the maximal endothelium-dependent relaxation by ACh were gradually reduced for up to 12 hours of warm ischemia (Table 2). There was a significant loss of endothelial function after more than 9 hours of warm ischemia, when compared with that in the fresh rat aorta. Maximal endothelium-independent relaxation induced for SNP and forskolin was not altered for up to 9 hours of warm ischemia (Table 2). However, effects of vasodilating agents on the rat aorta after 24 hours of warm ischemia were not measurable due to the low levels of contraction in response to NE (Table 2).

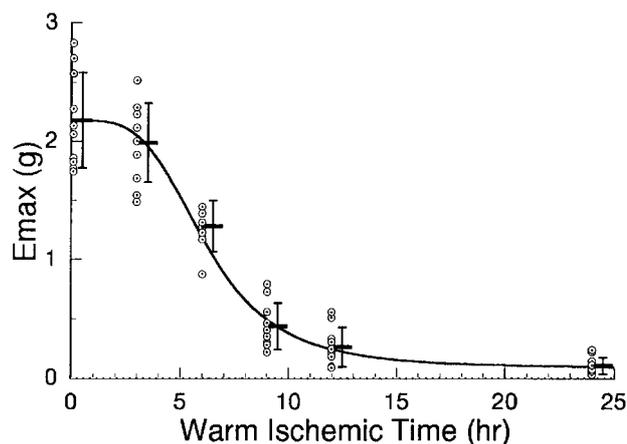


Fig. 1. Effect of the warm ischemia on response of the rat aorta to norepinephrine. Data were fitted to the equation $E_{\max} = a - bT^n / (T^n + c)$; E_{\max} : maximum effect (g); T : warm ischemic period (hours). The best fit was obtained with $a=2.18$, $b=2.09$, $c=945.8$, and $n=3.77$. The warm ischemic period that preserved more than 50% of the maximal response to norepinephrine observed at harvest immediately after death was 6.17 hours.

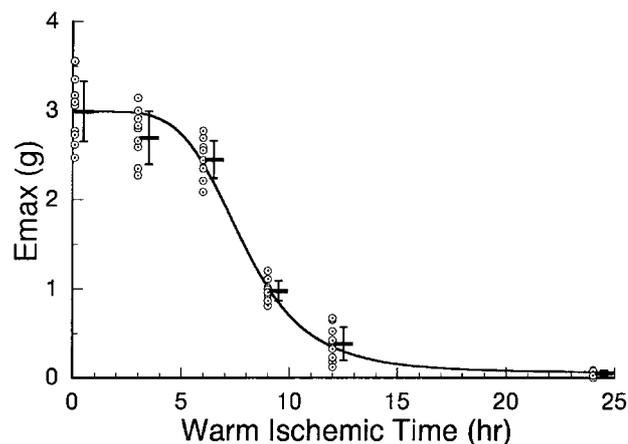


Fig. 2. Effect of the warm ischemia on the response to potassium chloride in the rat aorta. Data were fitted to the equation $E_{\max} = a - bT^n / (T^n + c)$; E_{\max} : maximum effect (g); T : warm ischemic period (hours). The best fit was obtained with $a=2.99$, $b=2.94$, $c=43559.3$, and $n=5.19$. The warm ischemic period that preserved more than 50% of the maximal response to potassium chloride observed at harvest immediately after death was 7.83 hours.

Calorimetric assay for cell viability

Cell viability as determined by WST-8 reduction gradually decreased as the warm ischemia prolonged (Table 3, Fig. 3). A significant negative correlation was found between the warm ischemic period (T : hours) and the cell viability (V : %) ($V=101.9-2.35T$; $r^2=0.96$; $p<0.0001$). There was a significant loss of mitochondrial dehydrogenase activity in the rat aorta between 9 and 24 hours after warm ischemia in comparison to that in control vessels ($p<0.0001$). Cellular metabolic activities were observed at more than 70% within 12 hours of warm ischemia.

Histological study

Typical histological aortic structure was retained in the

control tissues. Histological examination of all vessels after warm ischemia showed a greater loss of smooth muscle and endothelial cells than vessels examined immediately after death (Fig. 4). After 6 hours of warm ischemia, slight separation of the elastic fibers and focal vacuolation were seen sporadically in some specimens, but these changes were not widespread. After 9 hours of warm ischemia pyknotic nuclei and shrunken smooth muscle cells were seen, but these also were not widespread. Most vessels at 12 and 24 hours postmortem showed frequent and striking separation of structural elements, suggestive of widespread interstitial edema and increasing smooth muscle cell alterations. In these specimens, virtually all smooth muscle cells appeared dead

Table 2. Responses to relaxant agents in control rat aorta (H0) and 3 hours (H3), 6 hours (H6), 9 hours (H9), 12 hours (H12), and 24 hours (H24) after death

		H0	H3	H6	H9	H12	H24
ACh	pD ₂	7.10 ± 0.46	6.71 ± 0.38	6.73 ± 0.39	6.42 ± 0.10	6.17 ± 0.83*	n.d.
	E _{max} (g)	73.9 ± 14.8	65.5 ± 10.6	64.4 ± 7.0	50.9 ± 11.5**	42.8 ± 6.9*	n.d.
SNP	pD ₂	8.25 ± 0.24	8.11 ± 0.29	7.74 ± 0.37*	7.90 ± 0.14	7.89 ± 0.21**	n.d.
	E _{max} (g)	100.0 ± 0.0	100.0 ± 0.0	99.7 ± 0.4	98.2 ± 1.1	94.2 ± 5.4*	n.d.
Forskolin	pD ₂	7.93 ± 0.10	7.47 ± 0.39	7.31 ± 0.12*	7.04 ± 0.11*	7.06 ± 0.16*	n.d.
	E _{max} (g)	97.1 ± 0.1	98.4 ± 0.8	98.8 ± 0.4	97.9 ± 0.2	97.3 ± 0.2	n.d.

ACh: Acetylcholin, SNP: Sodium nitroprusside, pD₂: negative logarithm of the molar concentration producing 50% of maximal response, E_{max}: maximal relaxation expressed as percentage of maximal response to papaverine 100 μM.

Data were given as means ± SD. * $p<0.001$ vs. H0; ** $p<0.05$ vs. H0; n.d.: not determined.

Table 3. Cell viability as determined by WST-8 reduction in control rat aorta (H0) and 3 hours (H3), 6 hours (H6), 9 hours (H9), 12 hours (H12), and 24 hours (H24) after death.

	H0	H3	H6	H9	H12	H24
WST-8 reduction (%)	100.0 ± 1.2	96.2 ± 2.5*	89.6 ± 6.9*	81.6 ± 4.3**	71.5 ± 2.2**	45.8 ± 2.2**

Data were given as means ± SD. * $p < 0.001$ vs. H0; ** $p < 0.0001$ vs. H0.

with clearly pyknotic nuclei and shrunken torn of fibers (Figs. 5 and 6). After 24 hours of warm ischemia, not-clearly-stained nuclei of smooth muscle cells were widespread (Fig. 6).

Discussion

The present study was designed to ascertain the physiological, metabolic, and histological changes occurring in the rat aorta exposed to warm ischemia for varying periods. The present results showed that the mechanical functions of the rat aorta were well preserved for up to 6 hours of warm ischemia and that metabolic viability of cells was greater than 70% of the control for up to 12 hours after death. However, after more than 9 hours of warm ischemia, virtually irreversible histologic changes were seen.

The physiological function of the rat aorta after exposure to warm ischemia was evaluated by examining smooth muscle contractility and endothelium-dependent and -independent relaxation. Contractility was evaluated by NE and potassium chloride via receptor-mediated intracellular events and membrane depolarization with ex-

tracellular Ca^{2+} influx, respectively. Endothelial function was assessed by the ability of ACh to induce relaxation of the precontracted artery via the release of nitric oxide.¹³ SNP and forskolin were employed to examine their respective guanylate-cyclase- and adenylyl-cyclase-dependent pathways of relaxation. Contractile responses elicited by NE were significantly reduced after exposure to warm ischemia for more than 6 hours, but both receptor-dependent and -independent contractile functions were preserved at more than 50% of the maximal control response. On the other hand, relaxation elicited by SNP and forskolin tended to be well preserved for up to 12 hours. These findings suggest that the vascular smooth muscle contractile function through guanylate-cyclase and adenylyl-cyclase pathways was preserved relatively well for up to 6 hours, while the endothelium-dependent relaxation decreased in time-dependently along with the warm ischemic period. Since there was no attenuation in the relaxation response to SNP, results imply that the reduced relaxation in response to ACh was related to altered endothelial function, but not to an alteration in the guanylate-cyclase-dependent pathway of relaxation in the vascular smooth muscle. Histological examination revealed that warm ischemia led to a loss of smooth muscle

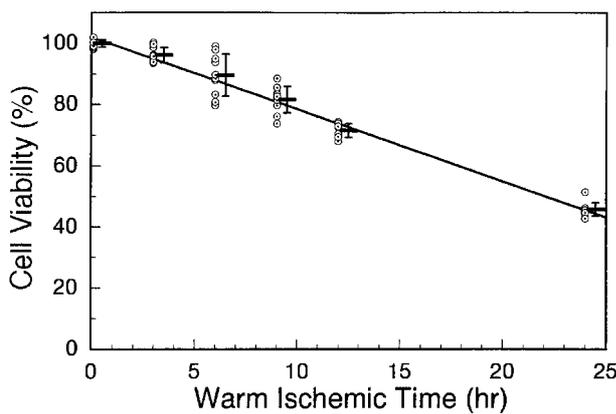


Fig. 3. Probability of cell viability as determined by WST-8 reduction related to the warm ischemic period. A significant negative correlation was found between the warm ischemic period (T : hours) and cell viability (V : %) ($V=101.9-2.35T$; $r^2=0.96$; $p < .0001$). Results were expressed as percentages of the control value.

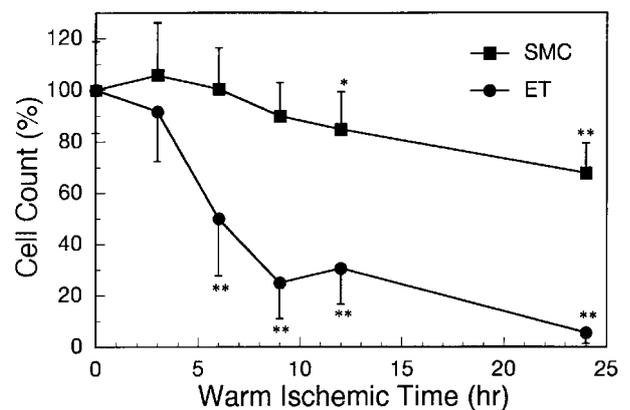


Fig. 4. Numbers of smooth muscle and endothelial cells in the rat aortic tissue after warm ischemia. Results were expressed as percentages of the control value. SMC: smooth muscle cells; ET: endothelial cells; * $p < 0.05$; ** $p < 0.0001$ vs the control (H0) group.

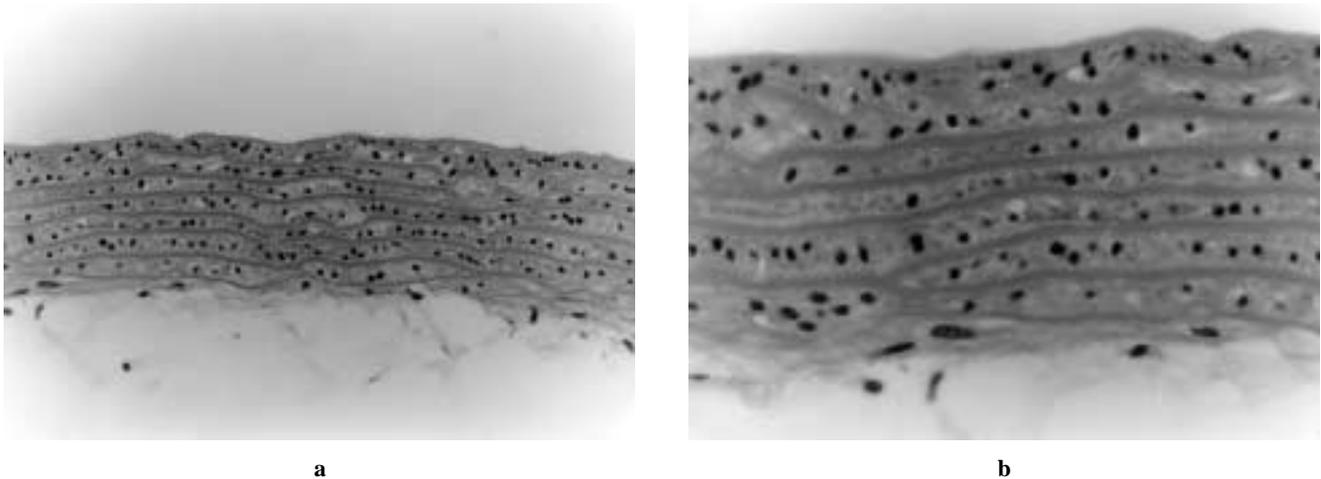


Fig. 5. Microscopic appearance of the aorta segment harvested 12 hours postmortem. Pyknotic nuclei and shrunken smooth muscle cells were seen. [Hematoxylin and eosin staining, original magnification $\times 100$ (a) and $\times 200$ (b)]

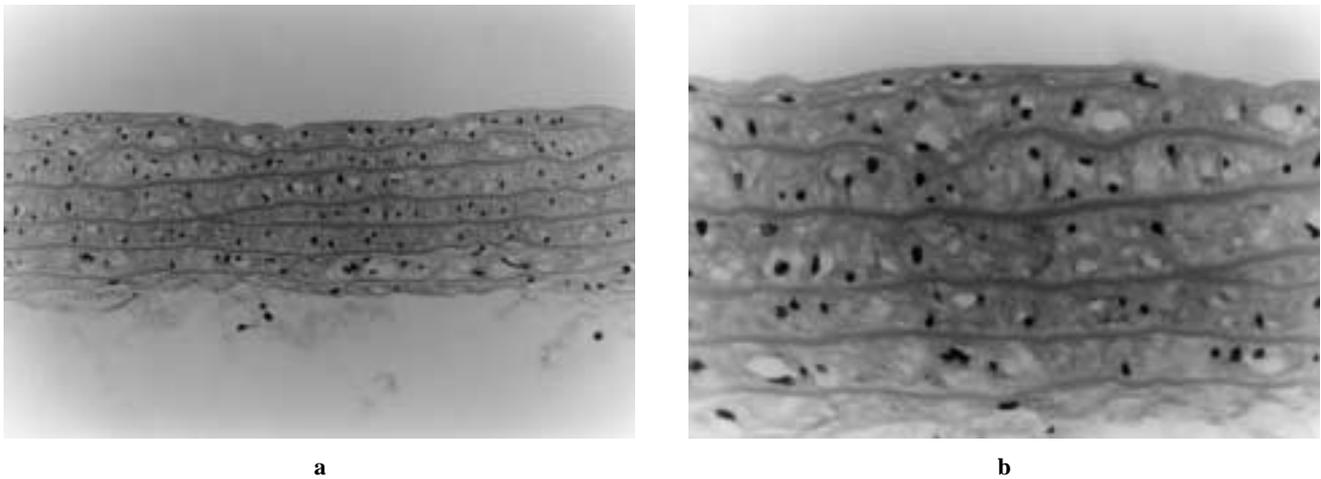


Fig. 6. Microscopic appearance of the aorta segment harvested 24 hours postmortem. In all cases, pyknotic or not-clearly-stained nuclei of smooth muscle cells were seen and were widespread. [Hematoxylin and eosin staining, original magnification $\times 100$ (a) and $\times 200$ (b)]

and endothelial cells. However, it was not clear whether the reduced response to ACh would be due to injured endothelial cells, loss of endothelial cells, or both.

The tetrazolium salt reduction technique involves use of quantitative assays to evaluate mitochondrial dehydrogenase enzyme activity within living cells. Loss of the mitochondrial dehydrogenase activity is indicative of irreversible cellular death and tissue infarction.^{14,15} 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl tetrazolium bromide (MTT) is widely used for this type of assay. However, we used an alternative of MTT, i.e., WST-8, since this compound produces a highly water soluble formazan dye, which makes the assay procedure easier to perform.^{16,17} The 70% cell viability observed for up to 12 hours of warm ischemic time was consistent with pre-

vious reports on cardiac valves.³⁻⁶ In the present study, however, there was a discrepancy between mechanical function of the vascular strip and cell viability after warm ischemia. The cell viability assay examines only specific aspects of cell function, but it does not always reflect functional capacities of the various types of cells that govern tissue viability. Even though warm ischemia induced irreversible damage to surface receptors, calcium channels, membrane permeability, and/or intracellular contractile proteins, mitochondrial dehydrogenase activity would be preserved.

Unimplanted grafts, after 24 hours of warm ischemia, have shown a greater degree of cell autolysis and loss of cohesiveness between the vessel wall layers than fresh grafts.¹¹ Our histological examination at both 12 and 24

hours after warm ischemia also showed irreversible changes such as pyknosis of the smooth muscle cells and separation and tearing of the elastic fibers. It remains unexplained whether these histological alterations would be caused by autolysis or other mechanisms.

Thus, present experiments showed that there was a progressive loss of physiological function and cell viability in the rat aorta along with the warm ischemic period from donor death to graft harvest. However, present findings suggest that a period of up to 6 hours of warm ischemia would be acceptable for preservation of tissue viability of the vessel prior to preimplantation processing or implantation.

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