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Matsumoto, Shinichi
Kuroda, Yoshikazu
Saitoh, Yoichi

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THE EFFECT OF PRESERVATION TEMPERATURE AND PERIOD ON RESUSCITATION OF THE ISCHEMICALLY DAMAGED CANINE PANCREAS DURING PRESERVATION BY THE TWO-LAYER (UNIVERSITY OF WISCONSIN SOLUTION / PERFLUOROCHEMICAL) METHOD

Shinichi MATSUMOTO, Yoshikazu KURODA, and Yoichi SAITOH

First Division, Department of Surgery,
Kobe University School of Medicine

INDEXING WORDS

pancreas transplantation; two-layer (UW/PFC) method; resuscitation; preservation temperature

SYNOPSIS

We have shown that 24 to 48 hour-preservation by the two-layer (University of Wisconsin solution (UW) /perfluorochemical (PFC)) method at 4°C resuscitates a canine pancreas subjected to 90 min of warm ischemia. However, it is necessary to shorten preservation period for resuscitation of the ischemically damaged pancreas in a clinical simultaneous pancreas-kidney transplantation. The purpose of this study was to clarify the effect of preservation temperature and period on the resuscitation of the ischemically damaged pancreas during preservation by the two-layer method.

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** Abbreviations: UW, University of Wisconsin solution; PFC, perfluorochemical; ATP, adenosine triphosphate; TAN, total adenine nucleotide; ECP, energy charge potential; OKY046, sodium (E)-3-[4-(1)-imidazolylmethl phenyl] propenate

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Authors' names in Japanese : 松本慎一, 黒田嘉和, 齋藤洋一

First of all, we examined the possibility of resuscitation of the ischemically damaged pancreas during short-term preservation by the two-layer method. After 90 minutes of warm ischemia, canine pancreases were preserved by the two-layer method at 4, 20, or 37°C. In control group, the pancreas graft was autotransplanted without preservation. Graft viability was judged by graft survival after autotransplantation. Pancreas grafts subjected to 90 min of warm ischemia were not viable (0/5). At 4°C, 5 to 12 hr-preservation did not resuscitate the grafts (0/3 and 0/3 respectively). At 20°C, 3 and 5 hr-preservation resuscitated the grafts (3/5 and 5/5 respectively), although 1 and 8 hr-preservation were not successful (0/3 and 0/3 respectively). At 37°C, all the grafts were not resuscitated irrespective of preservation period. It was clear that the ischemically damaged pancreas was resuscitated during shot-term preservation by the two-layer method only at 20°C.

Secondly, we measured tissue adenine nucleotide levels by high performance liquid chromatography and pancreatic tissue perfusions using H2 clearance technique on reperfusion and examined the viability of vascular endothelium by nuclear trypan blue staining to make clear the necessary conditions for resuscitation of the ischemically damaged pancreas at 20°C.

ATP tissue levels in one hr-preserved grafts were 2.55 ± 0.38 μ mol/g dry weight and were significantly lower compared with the levels in 5 and 8 hr-preserved grafts, 9.40 ± 2.09 ($P<0.01$) and 7.37 ± 1.06 μmol/g dry weight ($P<0.01$) respectively. On the other hand, nuclear trypan blue uptakes of endothelial cells in 8 hr-preserved grafts were 37.6 ± 11.6% and were significantly higher than 1 hr- and 5 hr-preserved grafts 5.6 ± 4.5 ($P<0.01$) and 5.0 ± 3.0% ($P<0.01$) respectively. As a consequence, pancreatic tissue perfusions in 8 hr-preserved grafts, 31.0 ± 3.5 ml/min/g, were significantly lower than 1 hr- and 5 hr-preserved graft 72.0 ± 11.6 ($P<0.01$), 63.9 ± 13.3 ml/min/g ($P<0.01$) respectively. However, thromboxane A2 synthesis inhibitor (OKY046 0.1mM/L) decreased the percentage of trypan blue uptake (8.2 ± 3.6%) without interfering ATP synthesis (8.44 ± 0.92 μmol/g dry weight) in 8 hr-preserved pancreas and tissue perfusions after reperfusion were dramatically improved (99.6 ± 11.8 ml/min/g). As a result the ischemically damaged pancreas was resuscitated (4/5, 80%). It was suggested that 1hr-

preservation was not enough to synthesize ATP, which was essential to repair damaged cells, although vascular endothelial cells were maintained. Eight hr-preservation incurs endothelial cell damage although ATP tissue levels were maintained and consequently microcirculation was disturbed at reperfusion but OKY046 protects endothelial cells against preservation / reperfusion injury. As a consequence, the ischemically damaged pancreas was resuscitated.

We conclude that short-term (3 to 8 hr) preservation at 20°C by the two-layer method with OKY046 accelerates ATP synthesis, which is essential for repairing damaged cells and protects microvascular endothelial cells. This makes it possible to resuscitate the canine pancreas graft subjected to 90 min warm ischemia. This method holds promise for pancreas-kidney transplantation from cardiac arrest donors.

INTRODUCTION

Resuscitation of ischemically damaged pancreas is important to enlarge the donor pool using pancreas from cardiac arrest donor. Recently we have shown that canine pancreases subjected to 90 min of warm ischemia are resuscitated during preservation at 4°C by the two-layer (University of Wisconsin solution (UW) /perfluorochemical (PFC)) method for 24-48 hrs.[15, 16] As the kidney is transplanted first and then the pancreas is transplanted in clinical simultaneous pancreas kidney transplantation[22] the pancreas grafts has to be resuscitated before kidney transplantation is accomplished. Therefore, it is necessary to shorten preservation period for resuscitation of the pancreas graft. During preservation by the two-layer (UW/PFC) method, ATP is synthesized within ischemically damaged pancreas via direct phosphorylation of adenosine which is contained in UW.[14, 20] Since ATP has an essential role in supplying energy for processes that repair damaged cells[18] it seems reasonable to suppose that synthesized ATP is used effectively to resuscitate ischemically damaged pancreas during preservation. Furthermore, as ATP synthesis during preservation by the two-layer method is an enzymatic reaction[20] it seems likely that increasing preservation temperature makes ATP synthesis faster and shorten the time necessary for resuscitation of ischemically damaged pancreas,

although the use of higher temperature incurs the risk of greater vascular injury.[21]

The purpose of this study is to examine the effect of preservation temperature and period on the resuscitation of the ischemically damaged canine pancreas graft during preservation by the two-layer method.

MATERIALS AND METHODS

Mongrel dogs both sexes weighing 12-18 kg were used in this study. Perfluorodecaline, one of the PFCs, was the kind gift of Dr. K. Yokoyama (The Green Gross Corporation, Osaka, Japan).
OKY046 was the kind gift from Ono Pharmaceutical Co., Ltd. (Osaka, Japan.)
UW was purchased from Du Pont Critical Care (Waukegan, IL).
Chemicals were from Sigma Co., Ltd. (St Louis, MO)

Operation procedures: Anesthesia was induced and maintained with sodium pentobarbiturate (25 mg/kg weight). After laparotomy, a left lobectomy of the pancreas with the splenic artery and vein attached was meticulously performed, followed by splenectomy. The unflushed segmental pancreas graft was left in the abdomen in the abdominal cavity for 90 min at body temperature. Then the pancreas graft was flushed out with 50 ml heparinized UW (1,000 units /50 ml UW) kept at preservation temperature through the splenic artery and preserved according to the experimental protocol. After preservation, the pancreas graft was autotransplanted in the neck as described previously[17] excising the remainder of the pancreas at the time of autotransplantation. After operation, the dogs received saline with 10% glucose (30 ml/kg weight) and parenteral penicillin (25 mg/kg weight) for three days. After three days, standard kennel diets were given.

Assessment of the graft viability : Graft viability was judged by graft survival following autotransplantation. A K-value of IVGTT more than 1.0 at two weeks after transplantation[11] or maintenance of normoglycemia at least five days after transplantation[1] was considered graft survival.

Measurement of adenine nucleotides: High-performance

liquid chromatography on a reversed column, CLC-ODS (6 x 150 mm) purchased from Shimazu manufacturing Co., Ltd (Tokyo, Japan), which was equilibrated with 100 mM sodium phosphate buffer (pH 6.0) containing 1.0% methanol, was employed to separate the quantitate ATP, ADP, AMP. Total adenine nucleotide (TAN) was calculated as follows: ATP + ADP + AMP. Energy charge potential (ECP) was calculated as follows: (ATP + ADP x 0.5) / TAN.[2)]

Preparation of tissue extracts: At the end of preservation, a part of pancreas was rapidly frozen with bronze tongs in liquid nitrogen, lyophilized overnight, and kept at -80 °C until analysis. The dry tissue was ground to a powder using a mortar and pestle. The dry tissue powder was weighed (200 mg) and homogenized in 3 ml ice cold 0.5 N perchloric acid. The precipitated proteins was removed by centrifugation, and 500 μl of supernatant was neutralized by the additions of 50 μl 1.0 KOH and 50 μl Tris. Following centrifugation, 10 μl of supernatant was injected into HPLC for analysis. Tissue adenine nucleotide levels were measured in group 2 and control group.

Perfusion experiments: Pancreas grafts subjected to 90 min warm ischemia and preserved by the two-layer method were used for perfusion experiments. Pancreas grafts were perfused via splenic artery with Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C) saturated with a 95% O2: 5% CO2 mixture in a nonrecirculating system during the first 4 min at flow rates of 1 ml/g/min followed by perfusion at rates of 2 ml/g/min for 4 min. After 8 min, 200 μM trypan blue was added to the perfusion medium, and after 10 more min the pancreases were fixed with 2% paraformaldehyde, 2% glutaraldehyde for 5 min. Pancreases were embedded subsequently in paraffin, sectioned and stained. Staining with hematoxylin and eosin allowed quantitation of non-parenchymal cells in three randomly selected fields of 5 sections from each pancreas. Staining with eosin alone permitted determination of the number of trypan blue-positive cells in the same manner. Trypan blue uptake is a reliable marker of loss of cell viability.[4)] For each pancreas, trypan blue-positive vascular endothelial cell nuclei in eosin-counterstained sections were counted without knowledge of the identity of the experiment group in three different high power (400 x) fields. Total nuclei were

counted in the same way in hematoxylin-eosin stained sections The percentage of nuclear trypan blue uptake was calculated by dividing trypan blue positive nuclei per 15 fields by all nuclei per 15 fields[5, 6] in group 2 and control group.

Measurement of pancreatic tissue perfusions: Pancreatic tissue perfusions were measured with a PHG 203 flow meter (Unique Medical Co., Tokyo, Japan) using H2 clearance technique[3] before procurement and after 1hr , 2hr , 4hr of reperfusion in group 2 and control group.

Experimental protocol: Pancreas grafts exposed to 90 min period of warm ischemia were preserved by the two-layer (UW/PFC) method using UW solution at 4°C for 5, 12 and 24 hr (1A, 1B, and 1C respectively), at 20 °C for 1, 3, 5 and 8 hr (2A, 2B, 2C and 2D respectively) and at 37°C for 1, 3 and 5 hr (3A, 3B, and 3C respectively). In addition, thromboxane A2 synthesis inhibitor OKY046 was added to UW solution during preservation by the two-layer (UW+OKY046/PFC) method at 20°C for 8hr preservation (2E). In control, the grafts were immediately autotransplantation after 90 min of warm ischemia without preservation (Control group).

Data analysis: All values are expressed as means ± SD. Statistics were performed according to an analysis of variance combined with Fisher's test. Significance was accepted if $p<0.05$.

RESULTS

Graft survival: After 90 min of warm ischemia, the grafts did not survive (0/5, 0%) when the graft was immediately autotransplanted (Table I). 24hr-preservation at 4°C resuscitated the ischemically damaged pancreas and graft survival rates were 5/5, 100%, although 5 and 12 hr-preservations did not resuscitate the grafts. 3 and 5hr-preservations at 20°C made it possible to resuscitate the ischemically damaged pancreas and graft survival rates were 3/5, 60% and 5/5, 100% respectively, although 1 and 8 hr-preservations were not effective and graft survival rates were 0/3, 0% and 0/3, 0% respectively. While, addition of OKY046 made it possible to resuscitate the ischemically damaged pancreas during 8hr-preservation at 20°C (4/5 80%). In group

3, no grafts were resuscitated. It is clear that resuscitation of the ischemically damaged pancreas during short-term preservation by the two-layer method is achieved only at 20°C.

Energy metabolism: Tissue ATP levels were 1.41 ± 0.40, 2.36 ± 0.28, 9.40 ± 2.09, 7.82 ± 1.19 and 8.44 ± 0.92 in control and groups 2A, 2C, 2D and 2E respectively (Table Ⅱ). Tissue TAN levels were 4.53 ± 1.18, 5.90 ± 0.40, 16.12 ± 2.55, 12.49 ± 2.41 and 10.12 ± 0.42 in control and groups 2A, 2C, 2D and 2E respectively. Tissue ECP levels were 0.37 ± 0.06, 0.48 ±0.03, 0.67 ± 0.05, 0.74 ± 0.04 and 0.89 ± 0.05 in control and groups 2A, 2C, 2D and 2E respectively. Groups of 2B, 2C, 2D and 2E have significant higher tissue ATP, TAN, and ECP levels compared with control group. However, in group 2A tissue ATP, TAN and ECP levels were significantly lower compared with 2C.

Nuclear trypan-blue up take test: Trypan blue uptake is a reliable marker of loss of cell viability.[6] Percentage of nuclear trypan blue dying of vascular endothelial cells in control group was 17.3 ± 8.7% (Figure 1). In group 2A and 2C, the percentage were significantly decreased to 5.6 ± 4.5 ($P<0.01$) and 5.0 ± 3.0% ($P<0.01$) respectively. In group 2D the percentage significantly increased to 37.6 ± 11.6% compared with all groups ($p<0.01$). However, addition of OKY046(group 2E), the percentage were significantly decreased to 8.2 ± 3.6% compared with group 2D ($P<0.01$).

Microcirculation after transplantation: Pancreatic tissue perfusions before procurement were 48.6 ± 8.2 ml/min/100g. In control group, pancreatic tissue perfusions were 53.7 ±11.8, 44.7 ±10.5 and 49.7 ±13.3 ml/100g/min after 1 hr, 2 hr, 4 hr of reperfusion respectively. In group 2A, pancreatic tissue perfusions were significantly increased compared with control at 2 hr of reperfusion ($P<0.05$). In group 2C pancreatic tissue perfusions maintained the levels of groups 2A. However, in group 2D, pancreatic tissue perfusions were 31.0 ± 3.5, 28.5 ± 7.5 and 22.2 ± 6.8 ml/100g/min after 1 hr, 2 hr, 4 hr of reperfusion respectively (Table Ⅲ). These are significantly lower compared with 2A and 2C groups ($P<0.01$). In group 2E addition of OKY046 significantly improved tissue perfusions (99.6 ± 11.8, 97.1 ± 14.6, and 86.0 ± 8.8 ml/100g/min after 1 hr, 2 hr, 4 hr of reperfusion respectively)

compared with group 2D ($P<0.01$).

DISCUSSION

Shortage of brain death donor is serious problem in organ transplantation. One of the strategies of solving this problem is using grafts from cardiac arrest donor. In order to use grafts from cardiac arrest donor functional recovery of the graft from warm ischemically damage is essential. Recently we have shown that the two-layer method at 4°C for 24 to 48 hr allows tissue ATP synthesis in pancreatic tissue and resuscitates 90 min of warm ischemically damaged canine pancreas grafts.[15, 16] However, in clinical simultaneous pancreas-kidney transplantation, it is necessary to shorten the preservation period for resuscitation of the pancreas graft because the kidney is usually transplanted first and then the pancreas is transplanted.[22] Short to intermediate term (5 to 12 hrs) preservation by the two-layer (UW/PFC) method at 4°C did not resuscitate the ischemically damaged pancreas grafts. We chose preservation temperature 20°C because metabolic rate at 20°C is only 16% of the value at 37°C but three folds of the value at 4°C.[19] We have anticipated that this condition facilitates ATP synthesis essential for repairing damaged cells and lower temperature below 37°C is effective in protecting vascular endothelial injury due to ischemia[21] although the use of higher temperature incurs the risk of greater vascular injury.[21] At 20°C, 3 to 5 hr-preservation made it possible to resuscitate the ischemically damaged grafts, although the ischemically damaged grafts were not resuscitated irrespective of preservation period at 37°C. Secondly, to examine necessary conditions for resuscitation of the ischemically damaged pancreas at 20°C, we measured tissue adenine nucleotide levels, viability of vascular endothelium by nuclear trypan blue staining and pancreatic tissue perfusions on reperfusion. One hr-preservation is not enough to synthesize ATP which is essential for repairing damaged cells and therefore the grafts were not resuscitated. 3 hr-preservation at 20°C is marginal limit concerning synthesis of ATP and 5 and 8 hr-preservation at 20°C are enough to synthesize ATP but 8 hr-preservation at 20°C did

not resuscitate ischemically damaged grafts. As ATP tissue level reflects mainly ATP levels in parenchymal cells when considering quantitative proportion of parenchymal cells and non-parenchymal cells (vascular endothelium) we examined the viability of non-parenchymal cells using nuclear trypan-blue up take test. After 90 min of warm ischemia the percentage of nuclear trypan blue dying in endothelial cells was 17.3 ± 8.7%. During 1 to 5 hr preservation by the two-layer method at 20°C this percentage was significantly decreased. This shows that two-layer method at 20°C restores the viability of ischemically damaged pancreatic non-parenchymal cells during preservation. However after 8 hr preservation by the two-layer method at 20°C the percentage is significantly increased compared with all other groups suggesting that 8 hr-preservation incurs vascular endothelial cell damage. To examine the influence of endothelial damage on vascular microcirculation we measured pancreatic tissue perfusions after transplantation. One and 5 hour-preservation by the two-layer method at 20°C improved pancreatic tissue perfusions compared with control. However, pancreatic perfusions were significantly decreased after 8-hr preservation. It seemed reasonable to think that 8hr-preservation incurred vascular endothelial cells and consequently caused microcirculatory disturbance. As a result the grafts were not resuscitated irrespective of high tissue ATP. If endothelial cells were protected against preservation injury and vascular microcirculations on reperfusion were improved by some method, the ischemically damaged grafts might be resuscitated during 8 hr-preservation at 20°C. Recently, prostanoids such as prostacyclin (PGI) and thromboxane A2 (TXA) have been suggested to play an important role in preservation-induced injury.[8, 9, 10] TXA synthetase inhibitor OKY046 prevents a decrease of both the PGI / TXA ratio and pancreatic tissue perfusions.[12, 13] In fact, addition of OKY046 reduced the percentage of nuclear trypan blue dying in endothelial cells and improved tissue perfusions after reperfusion. As a result the grafts were resuscitated during 8 hr-preservation by the two-layer method at 20°C. 5 hr-preservation is in good balance between synthesis of ATP and maintains vascular endothelial cells to

protect pancreatic microcirculation after transplantation. The two-layer method at 37°C did not restore ischemically damaged pancreas irrespective of preservation time. We speculated that vascular endothelial cells were irreversibly injured during preservation at 37°C and the graft did not survive although ATP was maintained during preservation just like 8 hr-preservation at 20°C without OKY046. In an ischemically damaged pancreas, exogenous adenosine as a direct precursor of AMP, is essential for regenerations of ATP via purine salvage pathway catalyzed by adenosine kinase[7] during preservation by the two-layer method.[14] However we do not know how the two-layer method and OKY046 work with respect to prevention of the damage of endothelial cells. The specific features that make the endothelium susceptible to preservation and reperfusion-induced injury and the mechanism of protection offered by the two-layer method and OKY046 must be the focus of future studies because the understanding of the mechanism of protection may lead to further beneficial refinements of the two-layer method.

We conclude that short-term (3 to 8 hr) preservation at 20°C by the two-layer method with OKY046 accelerates ATP synthesis, which is essential for repairing damaged cells and protects microvascular endothelial cells. This makes it possible to resuscitate the canine pancreas graft subjected to 90 min warm ischemia. This method holds promise for pancreas-kidney transplantation from cardiac arrest donors.

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Table I. Functional success rates of canine pancreas grafts after 90 min warm ischemia and preserved by the two-layer method.

Group	Preservation method	Preservation temperature (°C)	Preservation Time (hr)	Survival rates(%)	
1A	UW/PFC	4	5	0/3	(0)
1B	UW/PFC	4	12	0/3	(0)
1C	UW/PFC	4	24	5/5	(100)
2A	UW/PFC	20	1	0/3	(0)
2B	UW/PFC	20	3	3/5	(60)
2C	UW/PFC	20	5	5/5	(100)
2D	UW/PFC	20	8	0/3	(0)
2E	UW+OKY046/PFC	20	8	4/5	(80)
3A	UW/PFC	37	1	0/3	(0)
3B	UW/PFC	37	3	0/4	(0)
3C	UW/PFC	37	5	0/3	(0)
Control	-	-	-	0/5	(0)

Table Ⅱ. Tissue ATP level, TAN level and ECP of canine pancreas grafts after 90 min of warm ischemia and preserved by the two-layer method.

Group	ATP(μmol/g dry weight)	TAN(μmol/g dry weight)	ECP
Control	1.41 ± 0.40[b]	4.53 ± 1.18[b]	0.37 ± 0.06[b]
2A	2.36 ± 0.28[b]	5.90 ± 0.40[b]	0.48 ± 0.03[ab]
2C	9.40 ± 2.09[a]	16.12 ± 2.55[a]	0.67 ± 0.05[a]
2D	7.82 ± 1.19[a]	12.49 ± 2.41[ab]	0.74 ± 0.04[a]
2E	8.44 ± 0.92[ab]	10.12 ± 0.42[ab]	0.89 ± 0.05[a]

[a] Significantly different from control group ($p<0.01$)

[b] Significantly different from group 2C ($p<0.01$)

Table Ⅲ. Tissue perfusion after reperfusion of canine pancreas grafts subjected to 90 min of warm ischemia and preservation by the two-layer method.

Group	Tissue perfusion after reperfusion (ml/min/100g)		
	1 hour	2 hour	4 hour
Control	53.7 ± 11.8	44.7 ± 10.5	49.7 ± 13.3[b]
2A	72.0 ± 11.6[b]	66.0 ± 11.2[ab]	64.1 ± 12.5[b]
2C	63.9 ± 13.3[b]	57.1 ± 4.4[b]	55.6 ± 1.1[b]
2D	31.0 ± 3.5	28.5 ± 7.5	22.2 ± 6.8[a]
2E	99.6 ± 11.8[ab]	97.1 ± 14.6[ab]	86.0 ± 8.8[ab]

[a] Significantly different from control group ($p<0.05$)

[b] Significantly different from group2D ($p<0.05$)

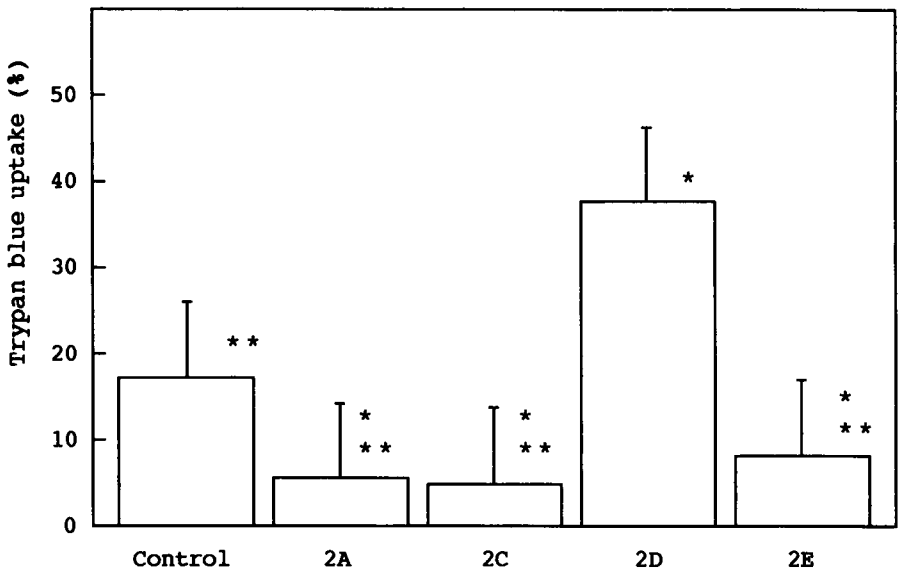

Figure 1. Percentage of trypan blue uptake of nuclei in endothelial cells infused with oxygenated Krebs Henseleit buffer at 37°C after 90 min of warm ischemia (control), after 90 min of warm ischemia and preservation by the two-layer (UW/PFC) method at 20°C for 1hr (2A), 5hr (2C), 8hr (2D) and with OKY046 for 8hr (2E).

Bar = mean ± SD of 15 fields