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THE MECHANISM OF ACTION OF THE TWO-LAYER COLD STORAGE METHOD IN CANINE PANCREAS PRESERVATION - PROTECTION OF PANCREATIC MICROVASCULAR ENDOTHELIUM -*

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INDEXING WORDS

pancreas transplantation ; pancreas preservation ; two-layer cold storage method ; microvascular endothelium

SYNOPSIS

We have demonstrated that oxygenation of a pancreas during preservation by a two-layer method leads continued ATP production to maintain cellular integrity and produces an extended period of preserved pancreatic viability. The aim of this study is to examine the effect of ATP vs. oxygenation per se on viability of nonparenchymal cell (vascular endothelium), using 2,4 dinitrophenol, an uncoupler of mitochondrial oxidative phosphorylation. Mongrel dogs of both sexes, weighing 12-18kg were used. Under general anesthesia, a left lobectomy of the pancreas was performed. The segmental pancreas graft was autotransplanted immediately (group 1 ; control) or after 48-hour

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Abbreviations : EC, Euro-Collins' solution; UW, University of Wisconsin solution; PFC, perfluorochemical; ATP, adenosine triphosphate.

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preservation (group 2 ; simple cold storage method with Euro-Collins' solution [EC], group 3 ; two-layer cold storage method using EC, group 4 ; two-layer cold storage method using EC with 0.2mM DNP), and the remainder of the pancreas was excised at the time of autotransplantation. Graft viability was judged from graft survival after transplantation. A K-value of intravenous glucose tolerance test more than 1.0 at 2 weeks after transplantation was considered graft survival. Tissue concentration of ATP was determined after preservation using high-performance liquid chromatography. Viability of vascular endothelium was examined using trypan-blue perfusion/fixation test after preservation. Nuclear staining by trypan blue in eosin-counterstaining sections was indicative of loss of cell viability. Pancreatic tissue perfusions, which reflect pancreatic microcirculation, were also measured using H_2 clearance technique after 30 to 240 min of reperfusion. Graft survial rates in groups 1, 2, 3 and 4 were 5/5, 0/4, 4/4 and 0/3 respectively. ATP tissue concentration was significantly higher in group 3 compared with group 2 (7.91 ± 1.21 [n=4] vs. 1.21 ± 0.31 [n=4] μmol/g dry weight, $p<0.01$). DNP caused a significant decrease in tissue ATP in group 4 (0.61 ± 0.07 [n=3] vs. 7.91 ± 1.21 [n=4] μmol/g dry weight, $p<0.01$). The percentage of nuclear trypan blue uptake of nonparenchymal cells in group 3 was significantly lower than group 2 (11.29 ± 3.71 [n=3] vs. 26.41 ± 1.66 [n=3] %, $p<0.01$), and DNP (group 4) increased trypan blue uptake (30.10 ± 4.08 [n=3] vs.11.29 ± 3.71 [n=3] %, $p<0.01$). Tissue perfusions after 2hr-reperfusion in group 3 were significantly higher than group 2 (68.64 ± 8.62 [n=5] vs. 45.56 ± 12.84 [n=5] ml/min/100g, $p<0.01$). Moreover, DNP (group 4) caused a significant decrease in pancreatic tissue perfusions (28.84 ± 9.09 [n=5] vs. 68.64 ± 8.62 [n=5] ml/min/100g, $p<0.001$). It was clear that the two-layer method (group 3) protected microvascular endothelium against cold ischemic damage and inhibition of ATP production using DNP (group 4) caused endothelial damage, microcirculatory disturbance after reperfusion and consequently loss of graft viability.

We conclude that microvascular endothelium of the pancreas graft is protected against cold ischemic injury by maintaining ATP tissue levels during preservation by the two-layer method. This is one of the mechanisms of action of the two-layer method in successful pancreas preservation.

INTRODUCTION

To reduce ischemic cell injury and tissue edema during simple cold storage of the pancreas, we developed a two-layer (Euro-Collins' solution [EC]/perfluorochemical [PFC]) cold storage method that continuously supplied sufficient oxygen to the pancreas during preservation.[9,11,12] We have also demonstrated that oxygenation of a pancreas during preservation by a two-layer method leads continued ATP production, which reflects mainly viability of pancreatic parenchymal cell, and produces an extended period of preserved pancreatic viability,[8,13] and 2,4 dinitrophenol, an uncoupler of mitochondrial oxidative phosphorylation, caused loss of graft viability with decrease of ATP tissue levels.[13] However, intact parenchymal cells can be of no value in the absence of circulation. Recently it has been reported that nonparenchymal cell (vascular endothelium) represents a major target for ischemia and reperfusion injury.[5,18] Damage of the vascular endothelium causes increased capillary permeability and edema, and decreases pancreatic tissue perfusion, resulting in early graft dysfunction. Thus, vascular endothelium could be the cellular compartment most sensitive to preservation damage.[7] The aim of this study is to examine the effect of ATP vs. oxygenation per se during preservation by the two-layer method on viability of vascular endothelium using DNP. Viability of vascular endothelium was determined by measuring nuclear dye uptake using Trypan-Blue perfusion/fixation test. Pancreatic microcirculation was also determined by measuring pancreatic tissue perfusions using H_2-clearance technique.

MATERIALS AND METHODS

Mongrel dogs of both sexes, weighing 12-18kg were used for the experiments. Perfluorodecaline, which is one of the PFCs, was a kind gift of Dr. K. Yokoyama (The Green Cross Corporation, Osaka, Japan).

Operation procedures

Anesthesia was induced and maintained with sodium pentbarbiturate (25mg/kg). After laparotomy, a left lobectomy of the pancreas with the splenic artery and vein attached was meticulously performed, followed by splenectomy. The segmental pancreas graft was washed with 50ml of cold heparinized EC (1000U/50ml EC) through the splenic artery and autotransplanted in the neck immediately or after 48-hour preservation, as described previously,[14] excising the remainder of the pancreas at the time of autotransplantation. After the operation, the dogs received saline with 10% glucose (30ml/kg) and parenteral penicillin (25mg/kg) for 3 days, standard kennel diets were given.

Preservation method

The two-layer cold storage method was performed as has been described previously.[11] The pancreatic graft was floated on the PFC, covered with EC solution in a styofoam box packed with ice and oxygenated throughout the storage period.

Functional studies

Blood glucose concentration was determined daily during the 1st postoperative week after autotransplantation and biweekly thereafter. Intravenous glucose tolerance tests (IVGTT) were performed at two weeks after transplantation. A K-value of IVGTT more than 1.0 at two weeks after transplantation was considered a viable graft.[1]

Measurement of ATP tissue concentration

ATP tissue concentration of pancreas grafts were measured after preservation using high-performance liquid chromatography as described previously.[12,13]

Experimental protocol

There were four experimental groups in which all dogs received segmental autografts that were autotransplanted immediately (group 1),

or were stored at 4℃ by the simple cold storage method with EC solution (group 2), the two-layer cold storage method using EC (EC/PFC) (group 3) and the two-layer cold storage method using EC with 0.2mM DNP (EC+DNP/PFC) (group 4) for 48hrs.

Experiment 1 - Trypan blue uptake of parenchymal cell and vascular endothelium after preservation -

Nuclear staining by trypan blue in eosin-counterstained sections is indicative of loss of cell viability.[3,4] Immediately after harvesting or after 48hr-preservation, the pancreas grafts were reperfused at 4℃ with nitrogen saturated-Krebs-Henseleit buffer. The initial flow rate of 1ml/min/g weight was increased to 2ml/min/g weight over 4min. After 8min of reperfusion, trypan blue (200 μM) was added to the perfusate. After a total of 18min of reperfusion, the pancreas grafts were fixed by infusion of 2% glutaraldehyde, 2% paraformaldehyde in perfusion buffer. For each pancreas graft, trypan blue-positive vascular endothelial cell nuclei in eosin-stained sections were counted. Total nuclei in endothelial cell were counted in H-E-stained sections. The percentage of nuclear trypan blue uptake was calculated by dividing trypan blue-positive nuclei per field by total nuclei per field.[5,6]

Experiment 2 - Pancreatic tissue perfusions after reperfusion

Pancreatic tissue perfusions, which reflects pancreatic microcirculation, were measured by using H_2-clearance technique [2,16] at laparotomy and after 30, 60, 120 and 240 min of reperfusion.

Experiment 3 - Effect of DNP on pancreas viability during 48hr preservation -

Pancreas grafts were preserved by simple cold storage in University of Wisconsin (UW) solution or UW with 0.2mM DNP for 48hrs. After transplantation, graft survival was examined.

Statistics

Data were expressed as mean±S.D. Statistical analysis was done using the Student's *t* test. A P value of <0.05 was considered significant.

RESULTS

Functional success rate in group 1, group 2 and group 3 was 5/5 (100%), 0/4 (0%) and 4/4 (100%), respectively. In contrast, after two-layer cold storage with EC+0.2mM DNP for 48hrs (group 4), functional success rate was 0/3 (0%) (Table I). DNP caused no toxicity in pancreas graft preserved for 48hrs in University Wisconsin solution (Table II). ATP tissue cncentration was significantly higher in group 3 compared with group 2 (7.91 ± 1.21 [n=4] vs. 1.21 ± 0.31 [n=4] μ mol/g dry weight, $p<0.01$). DNP again caused a significant decrease in tissue ATP in group 4 (0.61 ± 0.07 [n=3] vs. 7.91 ± 1.21 [n=4] μ mol/g dry weight, $p<0.01$) (Table I).

Table I. Effect of DNP on viability of pancreas graft during preservation by the two-layer method using EC for 48hr.

Group	Preservation Method	Functional grafts No. transplant	Success rate (%)	ATP tissue concentration (μ mol/g dry weight)
1	No preservation	5/5	100	4.44 ± 0.49
2	EC	0/4	0	1.21 ± 0.31 ┐*
3	EC/PFC	4/4	100	7.91 ± 1.21 ┤
4	EC+DNP/PFC	0/3	0	0.61 ± 0.07 ┘*

*$p<0.01$

Table II. Effect of DNP on viability of pancreas graft during simple cold storage in UW for 48hr.

Group	Preservation Solution	Functional grafts No. transplant	Success rate (%)
A	UW	4/4	100
B	UW+DNP	3/3	100

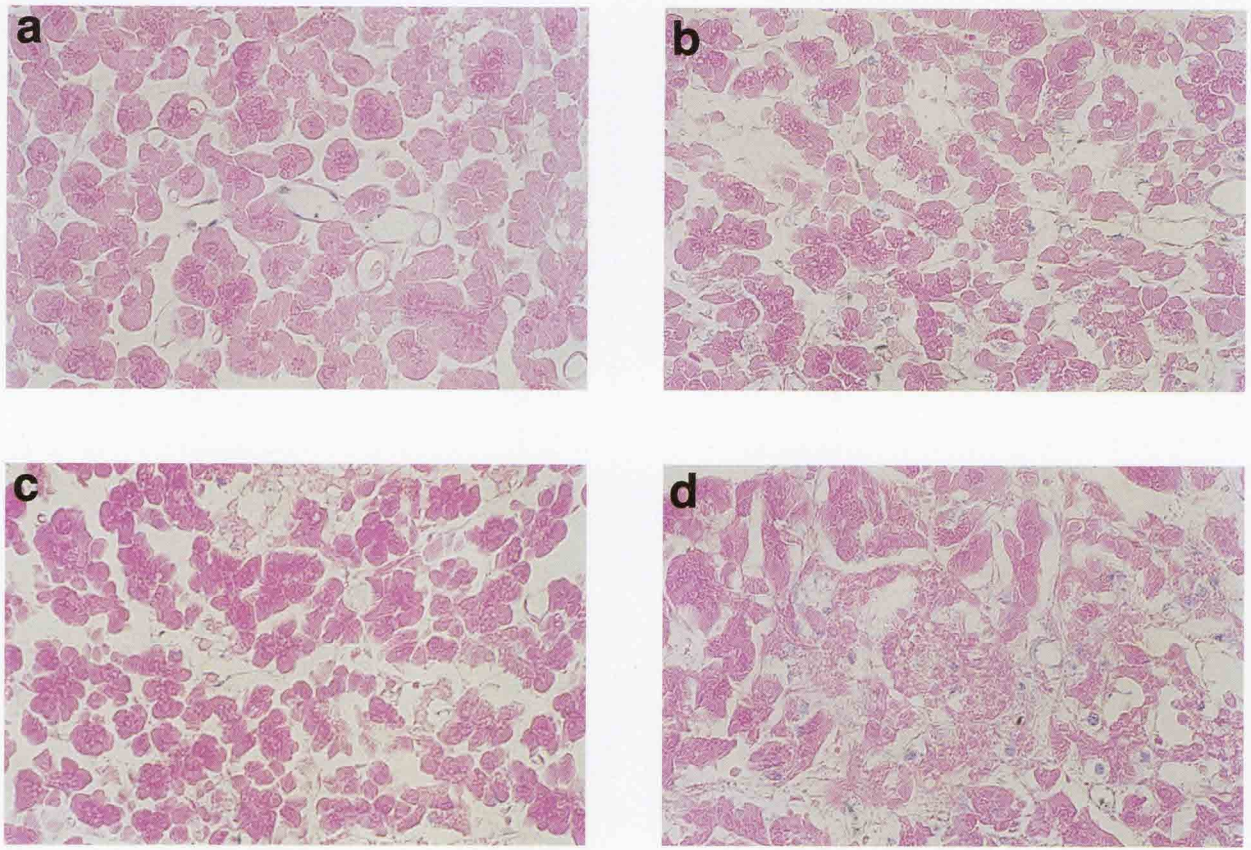

Figure 1. Light micrograph of trypan blue uptake in pancreas grafts infused with cold nitrogen-saturated Krebs Henseleit-bicarbonate buffer after harvesting (a), simple cold storage in EC (b), or preservation by the two-layer method using EC (c) or EC containing 0.2mM DNP (d) for 48hrs. (Eosin ; X400)

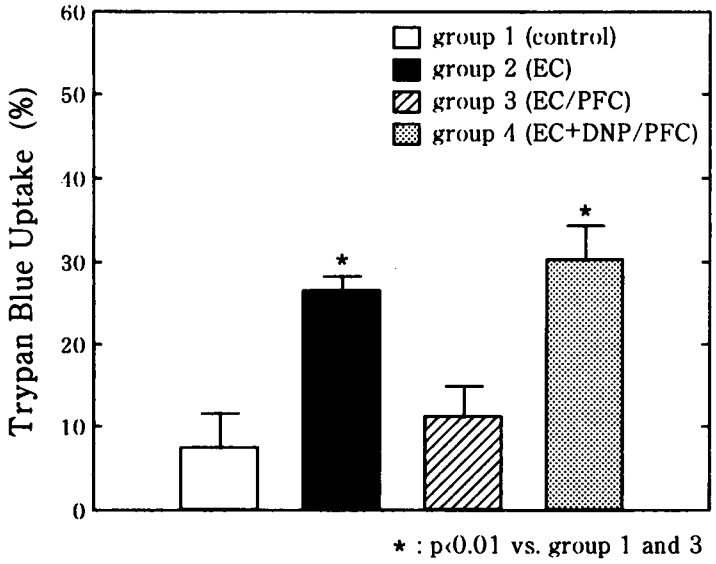

Figure 2. Percentage of trypan blue uptake in endothelial cells infused with cold nitrogen-saturated Krebs Henseleit-bicarbonate buffer after harvesting (group 1), simple cold storage in EC (group 2) or preservation by the two-layer method using EC (group 3) or EC containing 0.2mM DNP (group 4). Bars = mean ± S.D.

Nuclear dye uptake of vascular endothelium were 7.38±4.29% (n=3) in group 1 (Figure 1a), 26.41±1.66% (n=3) in group 2 (Figure 1b) and 11.29±3.71% (n=3) in group 3 (Figure 1c), suggesting endothelial cells were damaged during simple cold storage in EC for 48hrs but two-layer method (group 3) reduced this damage. However, in the presence of DNP (Figure 1d), nuclear trypan blue uptake was again increased to 30.10± 4.08% (n=3) (Figure 2). It was clear that microvascular endothelium of the pancreas graft was protected during preservation by the two-layer method and when ATP production was blocked by DNP, microvascular endothelium of the pancreas graft was damaged.

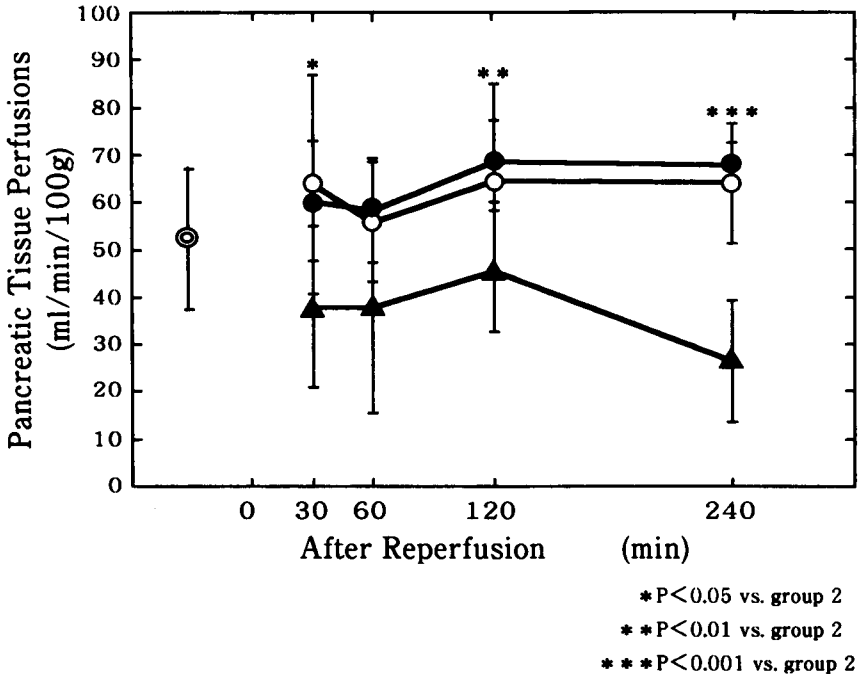

Figure 3. Pancreatic tissue perfusions at laparotomy (◎), in control (○ ; group 1), after simple cold storage in EC (▲ ; group 2) or preservation by the two-layer method using EC (● ; group 3) for 48hrs. Bars = mean ± S.D.

Pancreatic tissue perfusions at laparotomy were 52.12±14.81 ml/min/100g (n=20). In group 1, pancreatic tissue perfusions at 30, 60, 120 and 240 min after reperfusion were 63.62±23.02, 55.86±12.55, 64.46±20.61 and 64.00±12.57 ml/min/100g (n=5), respectively. Those in group 2 (37.84±16.97, 37.60±22.12, 45.56±12.84 and 26.50±12.80 ml/min/100g [n=5], respectively) were lower than in control. And at 240 min of reperfusion, pancreatic tissue perfusion in group 2 was significantly lower than in control ($p<0.01$). In contrast, those in group 3 (60.16±12.60, 58.36±11.02, 68.64±8.62 and 67.82±4.82 ml/min/100g [n=5], respectively) were as the same level as in group 1 (NS).

Moreover, pancreatic tissue perfusions in group 3 were significantly higher than those in group 2 at 30, 120 and 240 min of reperfusion ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) (Figure 3).

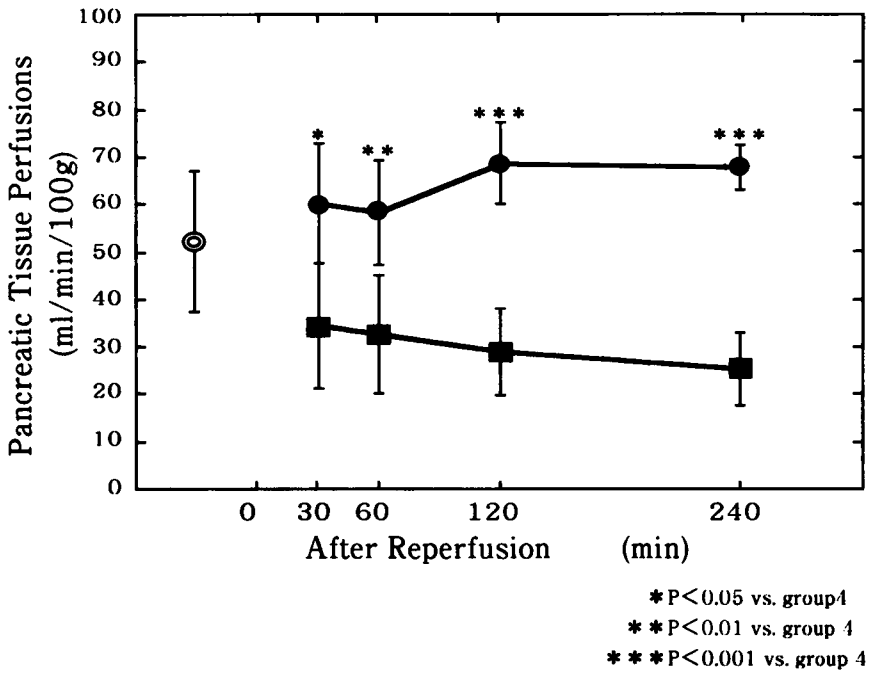

Figure 4. Pancreatic tissue perfusions in grafts preserved by the two-layer method using EC (● ; group 3) or EC containing 0.2mM DNP (■ ; group 4) for 48hrs. Bars=mean±SD

In the presence of DNP (group 4), pancreatic tissue perfusions at 30, 60, 120 and 240 min after reperfusion were 34.46±13.16, 32.58±12.33, 28.84±9.09, 25.28±7.73 ml/min/100g (n=5), respectively. And the value at 30, 60, 120 and 240 min of reperfusion in group 4 was significantly lower than that in group 3 (Figure 4). It was clear that microcirculation after reperfusion was protected by the two-layer (EC/PFC) cold storage method, but the microcirculation was disturbed when ATP synthesis was inhibited by DNP.

DISCUSSION

Nuclear staining by trypan blue in the eosin-counterstained sections was indicative of loss of liver cell viability.[3,4] Lemasters et al.[5] modified this technique to determine loss of cell viability during preservation by using nitrogen-saturated solution at 4℃. We applied this modified technique to determine endothelial damage of the pancreas during preservation. After preservation, pancreas grafts were infused with cold nitrogen-saturated solution and percentages of trypan blue uptake of endothelial cells were examined. Nuclear trypan blue uptake of endothelial cells was considerable after subsequent infusion of cold nitrogen-saturated solution, suggesting that endothelial cell damage occurred during cold preservation. The H_2-clearance technique has been applied successful to determine microcirculatory disturbance of several organs including the pancreas.[17,19] The method is simple and repeated measurements can be taken. Microcirculatory disturbance judged from pancreatic tissue perfusions using this method correlated with the endothelial cell injury determined by nuclear trypan blue uptake and graft survival after transplantation as reported by Kenmochi et al.[10] that tissue flow rate was a predictable index of isolated pancreatic graft viability. As nuclear trypan blue uptake of pancreatic microvascular endothelium reflects not only pancreatic vascular microcirculation but also graft survival after transplantation, trypan blue uptake provides a rapid and reliable means to evaluate endothelial cell damage. In this study, we demonstrated that vascular endothelial cells of the pancreas graft were sensitive to cold ischemia and lost extensively viability during 48hr simple cold storage in EC and consequently pancreatic microcirculation was disturbed after reperfusion. However, the two-layer method reduced endothelial cell damage and maintained pancreatic tissue perfusion. ATP is synthesized within pancreas graft using endogenous substrates mainly via mitochondrial oxidative phosphorylation when the graft is viable during preservation by the two-layer method.[12] However, DNP, an uncoupler of mitochondrial oxidative phosphorylation, inhibits ATP synthesis [13] and causes increase of trypan blue uptake (Figure 2), decrease of pancreatic tissue perfusion (Figure 4) and consequently loss

of graft viability (Table I). It is suggested that maintenance of ATP levels within the graft is essential for successful 48hr preservation of canine pancreas by the two-layer method, because DNP causes no toxicity in the graft (Table II) and did not causes decrease of pancreatic tissue perfusions in 48hr preserved grafts in UW (data not shown). Based on these results, it seems reasonable to think that ATP tissue levels during preservation by the two-layer method reflect not only the ability of parenchymal cells to synthesize ATP and graft survival after transplantation [15] but also the viability of endothelial cells.

We conclude that oxygenation of the pancreas during preservation by the two-layer method allows continued ATP production within the graft and protects vascular endothelium from cold ischemic injury. Consequently pancreatic tissue perfusions after reperfusion can be maintained. This is one of the mechanisms of action of the two-layer method in successful pancreas preservation.

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