Transplant Int (1992) 5 [Suppl 1]: S391–S394

TRANSPLANT
International

The mechanism of action of the two-layer (Euro-Collins' solution/perfluorochemical) cold storage method in canine pancreas preservation

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Abstract. To clarify the mechanism of action of a two-layer [Euro-Collins' solution (EC)/perfluorochemical (PFC)] cold storage method in the preservation of the pancreas, pancreatic viability and tissue concentrations of adenosine triphosphate (ATP) were examined in the canine model of pancreatic autotransplantation after preservation for 24 and 48 h by simple cold storage in EC (group 1), the two-layer, EC/PFC, method (group 2) and the two-layer, EC + 2, 4 dinitrophenol (DNP)/PFC, method (group 3). DNP is an uncoupler of oxidative phosphorylation. Maintenance of normoglycemia for at least 5 days after transplantation was considered a successful preservation. After preservation for 24 h, the functional success rates of groups 1, 2 and 3 were 100% (4/4), 100% (5/5) and 80% (4/5) respectively. One of five dogs in group 3 died of a cause unrelated to the pancreas. ATP tissue concentrations in group 2 were significantly higher than in group 1 (7.47 ± 0.47 μmol/g dry weight vs 1.41 ± 0.53 μmol/g dry weight, $P < 0.01$) and ATP tissue concentrations in group 3 were significantly lower than in group 2 (1.25 ± 0.37 μmol/g dry weight vs 7.47 ± 0.47 μmol/g dry weight, $P < 0.01$). It was apparent that ATP was not an essential factor for successful 24-hour preservation of the canine pancreas in EC because all the pancreatic grafts except one of five grafts in group 3 remained viable after preservation for 24 h, regardless of ATP tissue concentrations. On the other hand, after preservation for 48 h, the functional success rates for groups 1, 2 and 3 were 0% (0/4), 100% (4/4) and 0% (0/3) respectively. ATP tissue concentrations in group 2 were significantly higher than in group 1 (7.91 ± 1.21 μmol/g dry weight vs 1.21 ± 0.31 μmol/g dry weight, $P < 0.01$) and ATP tissue concentrations in group 3 were significantly lower than in group 2 (0.61 ± 0.07 μmol/g dry weight vs 7.91 ± 1.21 μmol/g dry weight, $P < 0.01$). It was clear that preservation of the pancreas for 48 h was unsuccessful by simple cold storage in EC (group 1) and the two-layer method (group 2) made preservation for 48 h possible by increasing ATP tissue concentrations. However, DNP (group 3) inhibited the synthesis of ATP and the effectiveness of the two-layer method for 48-hour preservation of the pancreas. It was clear that maintenance of high ATP tissue concentrations during preservation was essential for the successful preservation of the canine pancreas in EC by the two-layer method for more than 48 h. We concluded that an adequate supply of oxygen to the pancreas during preservation by the two-layer method led to sufficient production of ATP to maintain cellular integrity and permitted the improvement of pancreatic preservation.

Key words: Preservation of the pancreas – 48 h – Adenosine triphosphate – Perfluorochemical

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To reduce ischemic cell injury and maintain cellular integrity during cold preservation of the pancreas, we have developed a two-layer [Euro-Collins' solution (EC)/perfluorochemical (PFC)] cold storage method [1] that provides sufficient oxygen to the pancreas during preservation [2] and succeeds in preserving the canine pancreas in EC for 48–72 h [1, 3], although EC is effective only for preserving the canine pancreas for 24 h [1–4]. Since oxygen is one of essential metabolites depleted in the ischemic organ, it seems reasonable to suppose that the oxygenation of the pancreas during preservation by the two-layer method is essential in reducing ischemic cell injury and extending the preservation time. In addition, since the oxygenation of the pancreas by the two-layer method leads to the maintenance of high adenosine triphosphate (ATP) tissue concentrations [2] and there is direct correlation between high ATP tissue concentrations after preservation by the two-layer method and good post-transplant outcome [5] it is also reasonable to think that provision of sufficient oxygen to the pancreas by the two-layer method allows the continued production of ATP, maintains cellular integrity and prolongs the preservation time. But it is not clear whether maintenance of high ATP tissue concentrations

Table 1. Effect of DNP on viability of pancreatic graft after simple cold storage for 48 h

Group	Preservation solution	Preservation time (h)	Functional grafts No. transplants	Success rate[a] (%)
A_1	EC	24	5/5	100
A_2	EC + DNP	24	3/3	100
B_1	UW	48	4/4	100
B_2	UW + DNP	48	3/3	100

[a] Maintenance of normoglycemia for at least 5 days after transplantation was considered viable pancreas graft [7]

by the two-layer method is a primary determinant to success in extension of the preservation time or merely reflects well-preserved mitochondrial function during cold storage. To clarify this problem, we set up our study so that the pancreatic graft was sufficiently oxygenated but production of ATP was blocked by 2,4 dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, during preservation by the two-layer method and examined the viability of the pancreas following transplantation. The purpose of this study was to clarify the mechanism of action of the two-layer cold storage method in preservation of the canine pancreas.

Materials and methods

Mongrel dogs of both sexes, weighing 12–18 kg were used for the experiments. Perfluorodecaline, one of the PFCs, was a kind gift of Dr. K. Yokoyama (The Green Cross Corporation, Osaka, Japan). A Shim-pack was purchased from Shimazu Manufacturing. Chemicals were from Sigma.

Operation procedures. Anesthesia was induced and maintained with sodium pentobarbiturate (25 mg/kg). After the abdomen was opened, a left lobectomy of the pancreas with the splenic artery and vein attached was meticulously performed, followed by splenectomy. The segmental pancreatic graft was washed with 50 ml of cold heparinized EC (1000 units/50 ml EC) or cold heparinized EC containing 0.2 mM 2,4 dinitrophenol (DNP) through the splenic artery and preserved. After preservation, the pancreatic graft was washed with saline and autotransplanted in the neck as described previously [6], excising the remainder of the pancreas at the time of autotransplantation. After surgery, the dogs received saline with 10% glucose (30 ml/kg) and parenteral penicillin (25 mg/kg weight) for 3 days. After 3 days, standard kennel diets were given.

Functional studies. Blood glucose concentration was determined daily during the 1st postoperative week after autotransplantation and biweekly thereafter. Maintenance of normoglycemia for at least 5 days after transplantation was considered successful preservation [7].

Measurement of adenine nucleotides. High performance liquid chromatography on a reverse-phase column of Shim-pack CLC-ODS (6 × 150 mm), equilibrated with 0.1 M phosphate buffer, pH 6.0, containing 1% methanol was employed to separate and quantitate adenine nucleotides.

Tissue extraction method for adenine nucleotides. At the end of preservation, a piece of pancreas was rapidly frozen with bronze tongs in liquid nitrogen and kept at −70 °C until analysis. Lyophilized tissues were ground to a powder using a mortar and pestle. Dry tissue powder (200 mg) was then homogenized in 3 ml ice cold 0.5 N perchloric acid. The precipitated protein was removed by centrifugation, and 500 µl of supernatant was neutralized by the additions of 50 µl 1.0 N $KHCO_3$ and 50 N Tris. Following centrifugation, 10 µl of supernatant was injected into the high performance liquid chromatography for analysis.

Preservation method and experimental protocol. In experiment 1 we examined the cytotoxic effect of DNP on the viability of the pancreatic graft during simple cold storage for 48 h. The pancreatic grafts were preserved by simple cold storage in EC (group A_1, $n = 5$) or EC + 0.2 mM DNP (group A_2, $n = 3$) for 24 h or University of Wisconsin solution (UW) (group B_1, $n = 4$) or UW + 0.2 mM DNP (group B_2, $n = 3$) for 48 h and then autotransplanted. It has been well established that EC is effective in preserving the canine pancreas for 24 h [1–4], and UW is effective in preserving the canine pancreas for 72 h [5, 8]. In experiment 2 we examined the effect of DNP on the viability and ATP tissue concentration of the pancreatic graft after preservation by the two-layer method. The two-layer cold storage method was performed as has been described previously [1–3]. There were six experimental groups in which all dogs received segmental autografts that were stored at 4 °C by simple cold storage in EC (group 1) or the two-layer (EC/PFC) cold storage method (group 2) or the two-layer cold storage method plus DNP (EC + DNP/PFC) (group 3) for 24 h or 48 h.

Data analysis. All values were expressed as the mean ± SD. Differences between groups were tested by the Student's *t*-test.

Results

Cytotoxic effect of DNP on the pancreatic graft after simple cold storage for 48 h. After preservation by simple cold storage in EC (group A_1) or EC + DNP (group A_2) for 24 h or in UW (group B_1) or UW + DNP (group B_2) for 48 h, the pancreatic grafts were autotransplanted. The functional success rates of groups A_1, A_2, B_1, and B_2 were 100% (5/5), 100% (3/3), 100% (4/4) and 100% (3/3) respectively. It was clear that the concentration of DNP used here (0.2 mM) was not lethal for pancreatic grafts during simple cold storage for at least up to 48 h (Table 1).

Effect of DNP on the viability and ATP tissue concentrations of pancreas grafts after preservation by the two-layer method (Table 2). After preservation for 24 h, the functional success rates of groups 1, 2 and 3 were 100% (4/4), 100% (5/5) and 80% (4/5) respectively. One of five dogs in group 3 died of a cause unrelated to the pancreas. ATP tissue concentrations in groups 1, 2 and 3 after cold preservation were 1.41 ± 0.53 ($n = 4$) µmol/g dry weight, 7.47 ± 0.47 ($n = 5$) µmol/g dry weight and 1.25 ± 0.37 ($n = 4$) µmol/g dry weight respectively.

Although ATP tissue concentrations in group 2 were significantly higher than in group 1 ($P < 0.01$) and ATP tissue concentrations in group 3 was significantly lower than in group 2 ($P < 0.01$), all the pancreatic grafts except one of five grafts in group 3 remained viable for 24 h regardless of the preservation method. It was apparent that ATP was not an essential factor for successful 24-h preservation of the canine pancreas in EC. On the contrary, after preservation for 48 h, the functional success rates of groups 1, 2 and 3 were 0% (0/4), 100% (4/4) and 0% (0/3) respectively. It was clear that 48-h preservation of the canine pancreas was unsuccessful using simple cold storage with EC [1–4] and the two-layer method made preservation for 48 h possible [1–3]. However, DNP inhibited the effectiveness of the two-layer method in 48-hour pres-

Table 2. Effect of DNP on viability and ATP tissue concentrations of pancreatic grafts after preservation by the two-layer method

Group	Preservation method	Preservation time (h)	Functional grafts no. transplants	Success rate[a] (%)	ATP tissue concentration (μmol/g dry weight)
1	EC	24	4/4	100	1.41 ± 0.53
	EC	48	0/4	0	1.21 ± 0.31
2	EC/PFC	24	5/5	100	7.47 ± 0.47*
	EC/PFC	48	4/4	100	7.91 ± 1.21*
3	EC + DNP/PFC	24	4/5	80	1.25 ± 0.37
	EC + DNP/PFC	48	0/3	0	0.61 ± 0.07

[a] Maintenance of normoglycemia for at least five days after transplantation was considered viable pancreas graft [7]
* $P < 0.01$, compared with groups 1 and 3

ervation of the pancreas. ATP tissue concentrations in groups 1, 2 and 3 after cold preservation were 1.21 ± 0.31 ($n = 4$) μmol/g dry weight, 7.91 ± 1.21 ($n = 4$) μmol/g dry weight and 0.61 ± 0.07 ($n = 3$) μmol/g dry weight respectively. ATP tissue concentrations in group 2 were significantly higher than in group 1 ($P < 0.01$) and ATP tissue concentrations in group 3 were significantly lower than in group 2 ($P < 0.01$). Thus the two-layer method facilitated ATP synthesis in the pancreas during preservation [2, 4] and ATP production in the pancreas was inhibited by DNP. It was clear that maintenance of high ATP tissue concentrations during preservation was essential for successful preservation of the canine pancreas in EC by the two-layer method for more than 48 h.

Discussion

The simple cold storage of the canine pancreas with so-called "intracellular solutions", EC [4], Collins' solution [9–12] and Sack's solution [13] is effective for preservation for 24 h but not for preservation for more than 48 h. However provision of sufficient oxygen to the pancreas during preservation by the two-layer method [1, 2] has made it possible to preserve the canine pancreas with EC for up to 72 h [3]. Since the oxygenation of the pancreas by the two-layer method leads to the maintenance of high ATP tissue concentrations [2], this maintenance of high ATP tissue concentrations could have an essential role in maintaining cellular integrity and contributing to successful preservation of the canine pancreas in EC for more than 48 h by the two-layer method. On the other hand, high ATP tissue concentrations could be a secondary phenomenon and not essential for successful preservation of the pancreas for more than 48 h because the pancreas can be preserved for more than 48 h without any significant ATP present in simple cold storage with a UW [8] and a silica-gel filtered plasma [13, 14]. To clarify this problem, we set up our study so that the pancreas was sufficiently oxygenated but production of ATP was blocked by DNP during the two-layer storage period and we examined the viability of the pancreas following autotransplantation. The results showed clearly that maintenance of ATP tissue concentrations during preservation were essential for successful preservation of the pancreas in EC for more than 48 h by the two-layer cold storage method. But it remains unclear how ATP is utilized in maintaining cellular integrity during cold preservation by the two-layer method. This problem is currently being investigated. We concluded that the oxygenation of the pancreas during preservation by the two-layer method led to a production of ATP sufficient for maintaining cellular integrity and permitting the improvement of pancreatic preservation. In kidney preservation, the provision of sufficient oxygen by retrograde oxygen persufflation improves the preservation of canine kidneys that have suffered warm ischemia prior to preservation [15–17]. Whether the two-layer cold storage method might have a role in the preservation of the pancreas subjected to warm ischemia is inknown and is also under investigation.

Acknowledgement. This work was supported by a grant from the scientific research fund of the Ministry of Education, Japan.

References

1. Kuroda Y, Kawamura T, Suzuki Y, Fujiwara H, Yamamoto K, Saitoh Y (1988) A new simple method for cold storage of the pancreas using perfluorochemical. Transplantation 46: 457
2. Kuroda Y, Fujino Y, Kawamura T, Suzuki Y, Fujiwara H, Saitoh Y (1990) Mechanism of oxygenation of pancreas during preservation by a two-layer (Euro-Collins' sulution/perfluorochemical) cold storage method. Transplantation 49: 694
3. Kawamura T, Kuroda Y, Suzuki Y, et al. (1989) Seventy-two-hour preservation of the canine pancreas by the two-layer (Euro-Collins' solution/perfluorochemical) cold storage method. Transplantation 47: 776
4. Amiel J, Peraldi D, Bernard JL, LΦubiere R, Raymond G, Mouiel J (1984) Successful pancreatic allograft in dog after 24-hour cold ischemia. Transplant Proc 16: 126
5. Kuroda Y, Fujino Y, Morita A, Ku Y, Saitoh Y Correlation between high ATP tissue concentration and good posttransplant outcome of the pancreas graft after a two-layer cold storage method. Transplantation (in press)
6. Kuroda Y, Orita K, Iwagaki S, et al. (1987) A new technique or pancreatic exocrine diversion to the esophagus in canine segmental pancreatic autotransplantation. Transplantation 44: 583
7. Abouna GM, Heil JE, Sutherland DER, Najarian JS (1988) Factors necessary for successful 48-hour preservation of pancreas grafts. Transplantation 45: 270
8. Wahlberg JA, Love R, Landegaard L, Southard JH, Belzer FO (1987) 72-hour preservation of the canine pancreas. Transplantation 43: 5
9. Westbroek DL, de Gruyl J, Dijkhuis CM, et al. (1974) Twenty-four-hour hypothermic preservation: perfusion and storage of

the duct-ligated canine pancreas with transplantation. Transplant Proc 6: 319
10. de Gruyl J, Westbroek DL, Macdicken I, Ridderhof E, Verschoor L, van Strik R (1977) Cryoprecipitated plasma perfusion preservation and cold storage preservation of duct-ligated pancreatic allografts. Br J Surg 64: 490
11. Baumgartner D, Sutherland DER, Heil JE, Zweber B, Awad EA, Najarian JS (1980) Cold storage of segmental canine pancreatic grafts for 24 hours. J Surg Res 29: 248
12. Florack G, Sutherland DER, Dunning M, Zweber B, Najarian JS (1984) Function of segmental pancreas grafts subjected to warm ischemia prior to hypothermic preservation. Transplant Proc 16: 111
13. Toledo-Pereyra LH, Chee M, Condie RM, Najarian JS, Lillehei RC (1979) Forty-eight hour hypothermic storage of whole canine pancreas allografts: improved preservation with a colloid hyperosmolar solution. Cryobiology 16: 221
14. Florack G, Sutherland DER, Heil J, Zweber B, Najarian JS (1982) Long-term preservation of segmental pancreas autografts. Surgery 92: 260
15. Fischer JH, Czerniak A, Hauer U, Isselhard W (1978) A new simple method for optimal storage of ischemically damaged kidneys. Transplantation 25: 43
16. Ross H, Escott ML (1979) Gaseous oxygen perfusion of the renal vessels as an adjunct in kidney preservation. Transplantation 28: 362
17. Rolles K. Foreman J, Pegg DE (1984) Preservation of ischemically injured canine kidneys by retrograde oxygen persufflation. Transplantation 38: 102