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Hiraoka, K
Kuroda, Y
Saitoh, Y

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THE IMPORTANCE OF ADENOSINE METABOLISM IN ISCHEMICALLY DAMAGED CANINE PANCREAS DURING PRESERVATION BY THE TWO-LAYER COLD STORAGE METHOD

Kunihiko HIRAOKA, Yoshikazu KURODA, and Yoichi SAITOH

First Division, Department of Surgery
Kobe University School of Medicine

INDEXING WORDS

pancreas transplantation; two-layer cold storage method; warm ischemia, adenosine; adenine nucleotides

SYNOPSIS

The two-layer cold storage method using the University of Wisconsin solution (UW) allows continuous tissue adenosine triphosphate (ATP) production during the preservation period. UW contains 5mM of adenosine which has several mechanisms of action, however, its efficacy is controversial. In this study, at first, we investigated the metabolism of exogenous adenosine during preservation by the two-layer method in the canine pancreas graft subjected to 60 min warm ischemia. High concentration (> 5 mM) of adenosine in Euro-Collins'solution (EC) remarkably increased ATP and total adenine nucleotide (TAN) levels during 24 hr preservation by the two-layer method using EC. Furthermore, experiments with 5 mM

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** Abbreviations: UW, the University of Wisconsin solution; EC, Euro-Collins'solution; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ANs, adenine nucleotides; TAN, total adenine nucleotide; HPLC, high-performance liquid chromatography

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Authors' names in Japanese: 平岡邦彦, 黒田嘉和, 斎藤洋一

of [2-^{3}H] adenosine demonstrated that adenosine was metabolized and incorporated into adenine nucleotides (ANs). However, neither 5 mM of inosine, hypoxanthine nor adenine substituted for adenosine. It was clear that adenosine was directly phosphorylated and converted into ANs. Secondly, the effect of adenosine on the viability of the ischemically damaged pancreas graft was evaluated by graft survival following autotransplantation. The ischemically damaged pancreas grafts were preserved for 24 hours by simple cold storage in EC (group 1), EC with 5 mM of adenosine (group 2), the two-layer method using EC (group 3), EC with 5 mM of adenosine (group 4). Graft survival rates were 0/5(0%), 1/5(20%), 0/3(0%) and 4/5(80%) in groups 1, 2, 3 and 4 respectively. Adenosine was clearly effective on graft survival via recovering high tissue ATP and TAN levels only in case of the preservation by the two-layer method. We conclude that exogenous adenosine works as a substrate for ANs synthesis and is directly phosphorylated to ANs during preservation by the two-layer method. This is essential to repair damaged cells and maintain cellular integrity, making it possible to preserve ischemically damaged pancreas.

INTRODUCTION

Pancreas graft injury due to warm ischemia strongly affects posttransplant outcome.[11,12,19] Therefore, improving the viability of ischemically damaged pancreas are important to enlarge the donor pool using the pancreas graft from cardiac arrested donor.

In ischemia, cellular ATP level rapidly decreases and ATP is degradated to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and further to salvageable nucleosides, adenosine, inosine and hypoxanthine.[6] ANs are not able to diffuse across the cell membrane. However, once AMP has been converted to nucleosides, nucleosides can transverse the cell membrane and ATP synthesis at the time of restoration of a normal blood supply is delayed due to a loss of salvageable nucleosides.[16] Based on these facts, it is suggested that support of graft ANs levels during preservation may result in improved postoperative outcome.[7]

Recently we have demonstrated that the two-layer cold storage method using UW makes it possible to resuscitate the viability of ischemically damaged canine pancreas [8] and extend the preservation

time up to 48 hours.[9] The two-layer method allows continuous tissue ATP production during the preservation period and graft viability following transplantation is correlated with tissue ATP level at the end of preservation.[14] Furthermore, we have also showed that the two-layer cold strage method using EC, which does not contain adenosine, does not have the ability to preserve pancreas graft subjected to 60 min warm ischemia.[4] Therefore, it seems reasonable to suppose that adenosine is a key component in the preservation of ischemically damaged pancreas. Adenosine is one of the important components of UW and works as a substrate for the regeneration of ANs during the reperfusion period following cold storage.[17] However, many investigators have reported that adenosine has other potential mechanisms of action ; improvement of microcirculation,[5] inhibition of platelet aggregation [1] and protection of endothelial cell through the inhibition of oxygen radical formation by stimulated neutrophiles.[3] The purpose of this study was to investigate the role and effect of exogenous adenosine during preservation by the two-layer method in ischemically damaged canine pancreas.

MATERIALS AND METHODS

Mongrel dogs of both sexes (12-20 kg) were used in this study. Perfluorodecaline, which is one of perfluorochemicals and EC were kind gifts from Dr. K. Yokoyama (The Green Cross Corporation, Osaka, Japan). A reversed column, Sim-pack CLC-ODS (6 x 150 mm) was purchased from Shimazu manufacturing Co., Ltd. Tritiated adenosine ([2-^{3}H] adenosine) (962 GBq/mmol) was purchased from Amersham corp. and other chemicals were from Waco Co., Ltd.

Preperation of ischemically damaged pancreas graft

Anesthesia was induced and maintained with sodium pentobarbiturate (25 mg/kg weight). After laparotomy, the left lobe, with the splenic artery and vein attached, was harvested. The unflushed segmental pancreas graft was left in the abdominal cavity for 60 min.

Preperation 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 extraction. The dry tissue, which was ground to a powder, was weighed (200 mg) and homogenized in 3 ml of 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 of 1.0 N KOH and 50 µl of 0.2 M Tris (pH 7.4). Following centrifugation, the supernatant was kept until analysis.

Measurement of adenine nucleotides

At the end of preservation, tissue ANs levels were determined by high-performance liquid chromatography (HPLC) on tissue extracts. 10 µl of tissue extract was injected into HPLC on a reversed column, CLC-ODS, which was equilibrated with 100 mM of sodium phosphate buffer (pH 6.0) containing 1.0% of methanol.

Transplantation

After 60 min warm ischemia, the pancreas graft was flushed out with 50 ml of cold heparinized each preservation solution (1,000 units/50 ml of solution) through the splenic artery and preserved according to the experimental protocol. After perservation, the pancreas graft was autotransplanted in the neck [10] and the remaining pancreas was excised at the same time. After operation, the dogs received saline with 10% of 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 auto-transplantation. Either maintenance of normoglycemia for at least five days after autotransplantation or an intravenous glucose tolerance test K value of more than 1.0 two weeks after auto-transplantation was considered graft survival.

Experimental protocol

Tissue ATP and TAN levels at the end of preservation were measured by HPLC in all experiments.

Experiment 1 : The pancreas grafts were preserved for 24 hours either by the two-layer method using EC or simple cold storage in EC. Each EC contained varying concentrations of adenosine (0 to 10 mM).

Experiment 2 : After the pancreas graft was preserved for 48 hours by the two-layer method using EC with 5 mM of [2-^{3}H] adenosine (9.62 GBq/mmol), radioactivity in each fraction, which was eluted by HPLC, was determined by liquid scintillation counter.

Experiment 3 : The pancreas grafts were preserved for 24 hours by the two-layer method using EC or EC with various adenine nucleotide precursors, 5mM of adenosine, inosine, hypoxanthine or adenine.

Experiment 4 : The pancreas grafts were preserved for 24 hours by simple cold storage in EC (group 1) or EC with 5mM of adenosine (group 2), or the two-layer method using EC (group 3) or EC with 5mM of adenosine (group 4). Graft viability was assessed following autotransplantation in addition to tissue ATP and TAN levels.

Caluculation and statistical analysis

TAN was caluculated as the sum of ATP, ADP and AMP. All values were expressed as mean ± SD. Statistical analysis was performed by Student's t test. A p value of < 0.05 was considered significant.

RESULTS

Experiment 1: Effect of adenosine concentration on ANs synthesis.

While exogenous adenosine did not affect ANs synthesis during the simple cold storage in EC, tissue ATP level at the end of preservation by the two-layer method using EC increased depending on the concentration of adenosine in EC (Figure 1 A), and tissue TAN level also increased in parallel (Figure 1 B). More than 5 mM

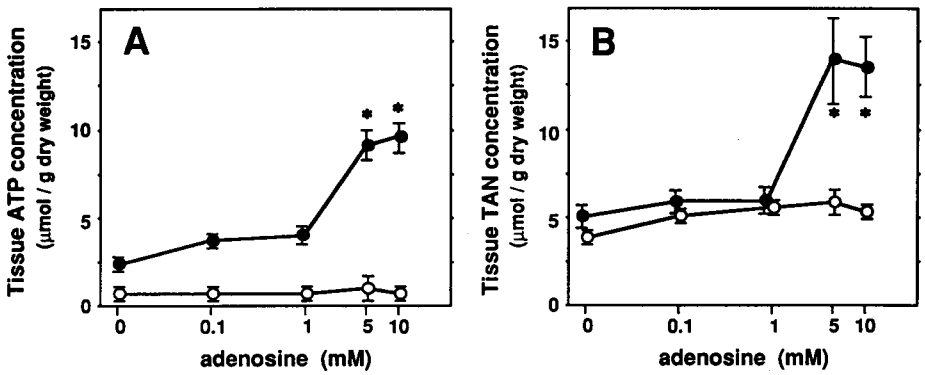

Fig. 1
Effect of adenosine concentration on tissue ATP levels (A) and TAN levels (B) in pancreas grafts subjected to 60 min warm ischemia. At the end of 24 hr preservation by the two-layer method using EC (●-●) or simple cold storage in EC (○-○), tissue ANs concentration was determined by HPLC. Results are given as mean ± SD of three experiments: * $P<0.05$ for comparison with the preservation without adenosine.

of adenosine was significantly effective in ANs synthesis ($p < 0.05$). Therefore, 5 mM of adenosine was utilized in all subsequent experiments.

Experiment 2: Distribution of radioactivity in pancreas.

At the end of 48 hr preservation by the two-layer method with 5mM of [2-^{3}H] adenosine, 79.4% of total radioactivity in the acid-soluble extract was detected in the all elutions and 53.2% of total radioactivity in the acid-soluble extract was incoporated into the adenine nucleotide fractions (ATP 42.2%, ADP 10.0%, AMP 1.0%). Much less radioactivity was found in the inosine fraction (6.8%), the hypoxanthine fraction (12.7%) and the adenine fraction (1.5%) (Figure 2). However, adenosine was not detectable by ultraviolet absorption and scintillation counter. The tissue TAN level which was regenerated in the pancreas graft during preservation by the two-layer method was 7.75 μmol/g dry weight and [2-^{3}H] adenosine incorporation into ANs was 6.67 μmol/g dry weight. There was not so much difference between these amounts. These results demonstrate that adenosine is a substrate for ANs regeneration.

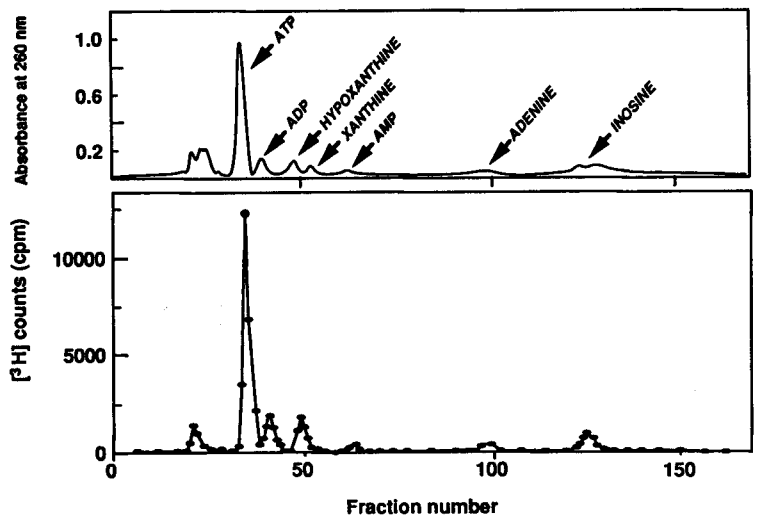

Fig. 2
Distribution of radioactive nucleotides and nucleosides. The pancreas graft subjected to 60 min warm ischemia was preserved by the two-layer method using EC with 5mM of [2-^{3}H] adenosine for 48 hours. Upper graph is the ultraviolet absorbance at 260 nm of the acid-soluble pancreas extract obtained by HPLC. Arrows represent the elution position of nucleotides and nucleosides. Bottom graph indicates the radioactivity of eluted fractions.

Experiment 3: Effect of various adenine nucleotide precursors on ANs synthesis.

5mM of inosine, hypoxanthine or adenine was exogenously supplied to EC in place of 5mM of adenosine during preservation by the two-layer method using EC and tissue ANs levels were measured at the end of 24 hr preservation. With adenosine, ATP and TAN levels increased to 8.94 ± 3.67 μmol/g dry weight and 14.28 ± 5.05 μmol/g dry weight respectively. However, without precursor, they remained stable at the low levels, 2.48 ± 1.25 μmol/g dry weight and 5.55 ± 1.76 μmol/g dry weight respectively. And with inosine, hypoxanthine or adenine, ATP and TAN levels did not increase (Figure 3). These results indicate that inosine, hypoxanthine and adenine do not substitute for adenosine, and are not intermediates in the conversion of adenosine to ANs.

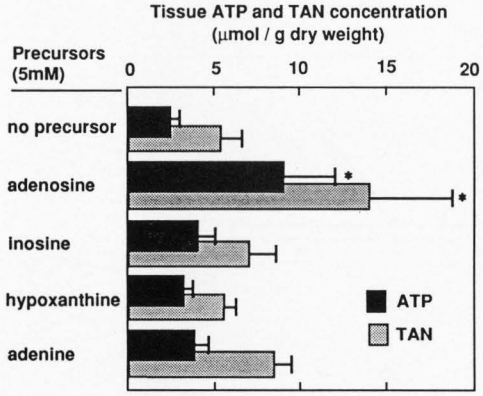

Fig.3
Effect of various precursors on the tissue ATP and TAN levels of pancreas grafts subjected to 60 min warm ischemia during 24 hr preservation by the two-layer method using EC. Results are given as mean ± SD of three experiments: * $P<0.05$ for comparison with preservation without precursor.

Table 1. Effect of adenosine on viability of pancreas grafts subjected to 60 min warm ischemia during 24 hr preservation

Group	Preservation method	Preservation solution	Graft survival rate (%)
1	Simple cold storage	EC	0/5 (0)
2		EC+adenosine	1/5 (20)
3	The two-layer method	EC	0/3 (0)
4		EC+adenosine	4/5 (80)

Table 2. Effect of adenosine on tissue ATP and TAN levels of pancreas grafts subjected to 60 min warm ischemia during 24 hr preservation

Group	Preservation method	Preservation solution	Tissue ATP level (μmol/g dry weight)	Tissue TAN level (μmol/g dry weight)
1	Simple cold storage	EC	0.43 ± 0.14	5.00 ± 0.75
2		EC+adenosine	1.01 ± 0.40	6.61 ± 1.03
3	The two-layer method	EC	2.79 ± 0.98	5.37 ± 1.30
4		EC+adenosine	8.36 ± 2.71*	12.86 ± 4.06**

* significantly different from group 1, group 2 and group 3 ($p<0.05$)
** significantly different from group 1, group 2 and group 3 ($p<0.05$)

Experiment 4: Effect of adenosine on viability of pancreas grafts.

Graft survival rates of autotransplantation after 24 hr preservation and ANs levels at the end of preservation are shown in Table 1 and Table 2. The graft survival rates of group 1 and group 2 were 0% and 20%, and there was no differences in ATP and TAN levels between group 1 and group 2 respectively. Adenosine did not affect the graft viability during preservation by the simple cold storage method. On the other hand, when the grafts were preserved by the two-layer method, adenosine had effects on survival rates and ANs levels. Without adenosine (group 3), survival rate was 0%, however, with adenosine (group 4), survival rate was increased to 80%. The ATP and TAN levels of group 4 were significantly higher than those of group 3 ($p < 0.05$). The two-layer method using EC with adenosine made it possible to preserve the ischemically damaged pancreas grafts for 24 hours with significantly higher tissue ATP and TAN levels.

DISCUSSION

We have recently demonstrated that the two-layer method using UW makes it possible to preserve and resuscitate the pancreas graft which was subjected to significant warm ischemia, and tissue ATP level at the end of the preservation will predict the posttransplant outcome of the graft.[8,9,14] UW contains 5 mM of adenosine, which works as a substrate for the regeneration of ATP during the reperfusion period.[17] In this study, high concentration of exogenous adenosine (5 mM to 10 mM) in EC had a remarkable effect on ANs synthesis during preservation by the two-layer method and tissue ATP and TAN levels increased in parallel with varying concentrations of adenosine. Studies with 5 mM of [2-^{3}H] adenosine demonstrated that adenosine was incorporated into ANs, which were regenerated during preservation by the two-layer method, and that is a evidence that adenosine works as a substrate for the regeneration of ANs. Furthermore, replacement of adenosine with other precursors of ANs, such as inosine, hypoxanthine or adenine, did not increase tissue ANs levels during the preservation period. These results indicate that adenosine is directly phosphorylated and converted to ANs during preservation by the two-layer method, and neither

inosine, hypoxanthine nor adenine was an intermediate in the conversion of adenosine to ANs (Figure 4).

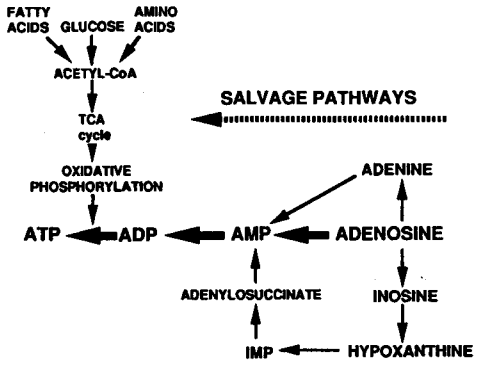

Fig.4
Adenosine metabolism during preservation by the two-layer method with adenosine.

Adenosine has numerous potential mechanisms of action besides energy substrate, (1) improvement of microcirculation [5], (2) inhibition of platelet aggregation [1] and (3) protection of endothelial cell through the inhibition of oxygen radical formation by stimulated neutrophiles.[3] Via these mechanisms of action it was shown that adenosine has some effects in minimizing or delaying preservation injury.[5,3] In this report, it seems unlikely that adenosine works for improvement of the grafts via these mechanisms of action, since the addition of adenosine during simple cold storage in EC did not have an effect on pancreas graft survival rates significantly. On the other hand, during preservation by the two-layer method using EC, exogenous adenosine in EC was clearly effective on graft survival rates via recovering high tissue ANs levels. These results show that adenosine works as a substrate for ANs synthesis and the two-layer method allows continuous tissue ATP production during the preservstion period, that is essential to repair damaged cells and maintain cellular integrity and consequently improves graft viability of ischemically damaged pancreas.

Several investigators have reported that nutritional support of donor liver may result in improved graft function via accumulation of glycogen in the liver.[2,13,15] On the contrary, recent report showed that fasting donor did not cause injury to the liver after warm or cold ischemia and liver glycogen did not appear to be important in

liver preservation.[18] Furthermore, we have demonstrated that nutritional condition of donor prior to procurement has no influence on viability of the canine pancreas graft.[4] The relationship between nutritional support of donor and graft survival are controversial. Therefore, metabolic intervention to affect ANs synthesis during preservation is important to improve and maintain the viability of graft and we showed the importance of adenosine metabolism during preservation by the two-layer method.

We conclude that provision of adenosine to ischemically damaged pancreas during preservation by the two-layer method allows ANs regeneration and ATP synthesis within the graft via direct phosphorylation of adenosine. Metabolic processes vital to repair damaged cell and maintain cellular integrity can be sustained, making it possible to preserve ischemically damaged pancreas.

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