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ORIGINAL ARTICLE

Yasuhiro Iwanaga
Yasuyuki Suzuki
Yoshikatsu Okada
Hiroshi Mori
Ippei Matsumoto
Masaaki Mitsutsuji
Yasuki Tanioka
Yasuhiro Fujino
Masahiro Tominaga
Yonson Ku
Yoshikazu Kuroda

Ultrastructural analyses of pancreatic grafts preserved by the two-layer cold-storage method and by simple cold storage in University of Wisconsin solution

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Y. Iwanaga · Y. Suzuki (✉)
I. Matsumoto · M. Mitsutsuji
Y. Tanioka · Y. Fujino · M. Tominaga
Y. Ku · Y. Kuroda
Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kobe University, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
E-mail: szk@med.kobe-u.ac.jp
Tel.: +81-78-3825925
Fax: +81-78-3825939

Y. Okada · H. Mori
The Second Department of Pathology, Osaka Medical College, Osaka, Japan

Abstract The two-layer cold storage method (TLM) using University of Wisconsin (UW) solution supplies sufficient oxygen to pancreatic grafts during preservation and extends pancreas preservation time to up to 96h in the canine model. Simple cold storage in UW (UWM) on the other hand, preserves canine pancreas grafts for up to 72h by preventing cell swelling, mainly because of its high osmotic pressure. The aim of this study is to analyze morphologically dog pancreatic grafts preserved by these two methods with their different mechanisms. Immediately after preservation of canine pancreata by TLM for 72h and 96h (group 1 and group 3, respectively), and by UWM for 72h and 96h (group 2 and group 4, respectively), tissue ATP levels were determined using high-performance liquid chromatography (HPLC), and detailed morphological analyses of intragraft components were performed using light- and electron microscopy. The mean areas of one mitochondrion and rough endoplasmic reticulum (RER) vacuolization were calculated by computer-graphic analyses using NIH image 1.62f soft. The tissue ATP levels were significantly higher in groups 1 and 3 than groups 2 and 4 ($P < 0.05$). Light microscopy demonstrated no marked difference among the 4 groups. By electron microscopy however, mitochondrial swelling and RER vacuolization were observed in acinar cells to various extents in the 4 groups. They were significantly more evident in group 2 than group 1 ($P < 0.05$), and in group 4 than group 3 ($P < 0.05$). In conclusion, TLM demonstrated excellent protection of intracellular organelles, mitochondria, and RER, up to 72–96h. Well-maintained graft ATP levels in TLM groups may result in maintaining the integrity of intracellular organelle membranes as well as cellular membranes.

Keywords Pancreas transplantation · Perfluorochemical · Two-layer cold-storage method · University of Wisconsin solution · Ultrastructural analyses · NIH image1.62f

Abbreviations *HPLC* high-performance liquid chromatography · *PFC* perfluorochemical · *RER* Rough endoplasmic reticulum · *TLM* Two-layer simple cold-storage method · *UW* University of Wisconsin · *UWM* Simple cold-storage method in UW solution

Introduction

It is important to minimize cold ischemic injury of pancreatic grafts in order to avoid delayed graft function, graft pancreatitis, thrombosis, and, possibly, acute- and chronic rejection, even if the graft has been successfully implanted. Simple cold storage in University of Wisconsin (UW) solution (UWM) has been the standard for organ preservation. It preserves canine pancreatic grafts for up to 72h, preventing cell swelling due to its high osmotic pressure [5, 18]. On the other hand, we have previously developed the two-layer cold-storage method (TLM) which combines perfluorochemical (PFC), which is a hyper oxygen carrier, with a balanced solution, such as Euro-Collins or UW solution for pancreas preservation [9]. This method successfully prolonged the preservation time to up to 96h in a canine experiment [2]. During preservation by the TLM, the pancreas is continuously oxygenated through PFC supplied with 95% oxygen, and consequently ATP is synthesized in the graft tissue [8, 9, 10]. Subsequently, we demonstrated that this generated ATP is used to drive a Na^+/K^+ pump to maintain cell integrity, hence the TLM physiologically prevents cell swelling [15]. The aim of this study is to analyze morphologically, by light- and electron microscopy, canine pancreatic grafts preserved by these two methods with their different mechanisms.

Materials and methods

Mongrel dogs of both genders, weighing 10–12 kg were used in this study. All animal experiments were conducted according to "Principles of laboratory animal care" (NIH publication No. 86–23, revised 1985) as well as "Guidelines for animal experimentation at Graduate School of Medical Sciences, Kobe University". Perfluorodecaline, which is a high oxygen carrier PFC, was a kind gift from Dr. K. Yokoyama (The Green Cross Corporation, Osaka. Japan). UW was purchased from Du Pont Critical Care (Waukegan, IL, USA).

Operative procedures

Anesthesia was induced and maintained with sodium pentobarbiturate (25mg/kg). After laparotomy through a midline incision, a left lobectomy of the pancreas attached to the splenic artery and vein was performed. The pancreatic graft was then flushed through the splenic artery with 50ml of UW solution and preserved according to the experimental protocol.

Preservation method

Canine pancreata were preserved by UWM or TLM (UW/PFC). TLM was performed as described previously [6, 7]. Since PFC is water immiscible, and the specific gravity of PFC is 1.95, PFC forms a separate phase from UW. During preservation, the pancreatic graft stayed on the surface of the PFC, covered with UW solution. Oxygenation (95% oxygen and 5% CO_2) was continued through the fritted glass to PFC throughout the storage periods. Temperature was maintained at 4°C on ice.

Experimental groups

This study included four groups, configured according to preservation methods and time. In groups 1 and 2, pancreatic grafts were preserved by TLM for 72 h ($n = 10$) and 96h ($n = 10$), respectively. In groups 3 and 4, the preservation was performed by UWM for 72h ($n = 10$) and 96 h ($n = 10$), respectively. Non-preserved grafts served as control.

Measurement of tissue ATP concentration

At the end of preservation, a sample of the pancreas was rapidly frozen with bronze tongs in liquid nitrogen, lyophilized overnight, and kept at –80 °C for further evaluation. Dry tissue was ground to powder using a mortar and pestle, and 200mg of powder was homogenized in 3ml of ice cold 0.5N perchloric acid. Precipitated proteins were removed by centrifugation, and 500μl of the supernatant was neutralized by adding 50μl of 1.0N KOH and 50μl of 0.2M Tris (pH 7.4). Following centrifugation, tissue ATP concentration was determined by high-performance liquid chromatography (HPLC). Ten microliters of the supernatant was injected onto HPLC and separated on a reverse phase column of CLC-ODS that had been equilibrated with 100mM of sodium phosphate buffer (pH6.0) containing 1.0% of methanol [19].

Light microscopy

A sample of the pancreatic graft was fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin. Specimens were examined by standard light microscopy.

Electron microscopy

Samples of graft tissue were removed and fixed in 3% glutaraldehyde that had been buffered with 0.1M s-collidine (pH7.4), cut into several pieces and further fixed for 3h in the same fixative, and postfixed with 1% osmium tetroxide for 1h at 4°C. The tissue blocks were dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were double-stained with 2% uranyl acetate and lead citrate, and examinded with a JEM-100SX electron microscope (JEOL, Tokyo, Japan) at 80kV. In acini, islets and endothelial cells, ultrastructural alterations of intracellular organelles including nuclei, rough endoplasmic reticula (RER), mitochondria and Golgi apparatus were compared.

Morphometry

Morphometric analyses at ultrastructural levels were performed on 20 electron micrographs, taken randomly from 20 blocks prepared per animal. The mean surface areas of one mitocondrion and vacuoler RER were analyzed with a Macintosh computer using NIH image 1.62f soft.

Statistical analysis

All values were expressed as mean ± SD. Statistical analysis was performed using the unpaired Student's t-test. $P < 0.05$ was considered significant.

Results

Tissue ATP concentrations of groups 1 and 3 were 14.28 ± 1.94 and 10.43 ± 3.89 (μmol/g dry weight), respectively. In sharp contrast, those of groups 2 and 4 were 1.42 ± 0.50 and 1.02 ± 0.15 (μmol/g dry weight), respectively. The concentrations were significantly higher in groups 1 and 3 compared to groups 2 and 4 ($P < 0.05$).

On light microscopy, pancreatic structure, cell size and shape were well preserved to similar extent in all 4 groups (photos not shown). No differences were noted among the 4 groups and the control.

On electron microscopy, no marked differences were observed in islets among the 4 groups and the controls (photos not shown). Endothelial cells had short cytoplasmic processes on the cell surfaces in all groups. No significant differences could be found in their cytoplasmic organelles or nuclei (photos not shown). In acinar cells, however, vacuoler RER were observed to have more intensity in groups 2 and 4 than in groups 1 and 3 (Fig. 1). Varying extents of mitochondrial swelling was also observed. It was slightly more prominent in groups 2 and 4 compared to groups 1 and 3 (Fig. 1). In addition, degenerative changes of zymogen granules were evident in groups 2 and 4, particularly in the areas close to the luminal surface, while no remarkable changes of zymogen granules could be seen in groups 1 and 3 (Fig. 2; only groups 3 and 4 shown). The electron density of zymogen granules was decreased by approximately 40% in group 2 (data not shown), and more than half of zymogen granules were vacuolated in group 4 (Fig. 2). No morphological change of the nuclei or Golgi apparatus was observed in this study.

The mean areas of one mitochondrion by computergraphic analyses in the control (no preservation), groups 1, 2, 3 and 4, were 0.20 ± 0.03, 0.24 ± 0.03, 0.29 ± 0.04, 0.30 ± 0.04 and 0.35 ± 0.05 (μm^2), respectively. Mitochondrial swelling increased in a time-dependent manner with both methods. However, it was significantly higher in group 2, compared to that of group 1, and in group 4, compared to that of group 3 ($P < 0.05$). It is notable that no significant difference was observed between group 1 and the controls (Fig. 3).

Morphometry on vacuoler RER revealed a more distinct difference between the two preservation methods. The mean areas in the control and groups 1, 2, 3 and 4 were 2.86 ± 0.65, 3.05 ± 0.01, 15.51 ± 3.25, 5.01 ± 1.32 and 21.53 ± 5.23 (μm^2), respectively. Vacuolization of RER was significantly less in TLM groups 1

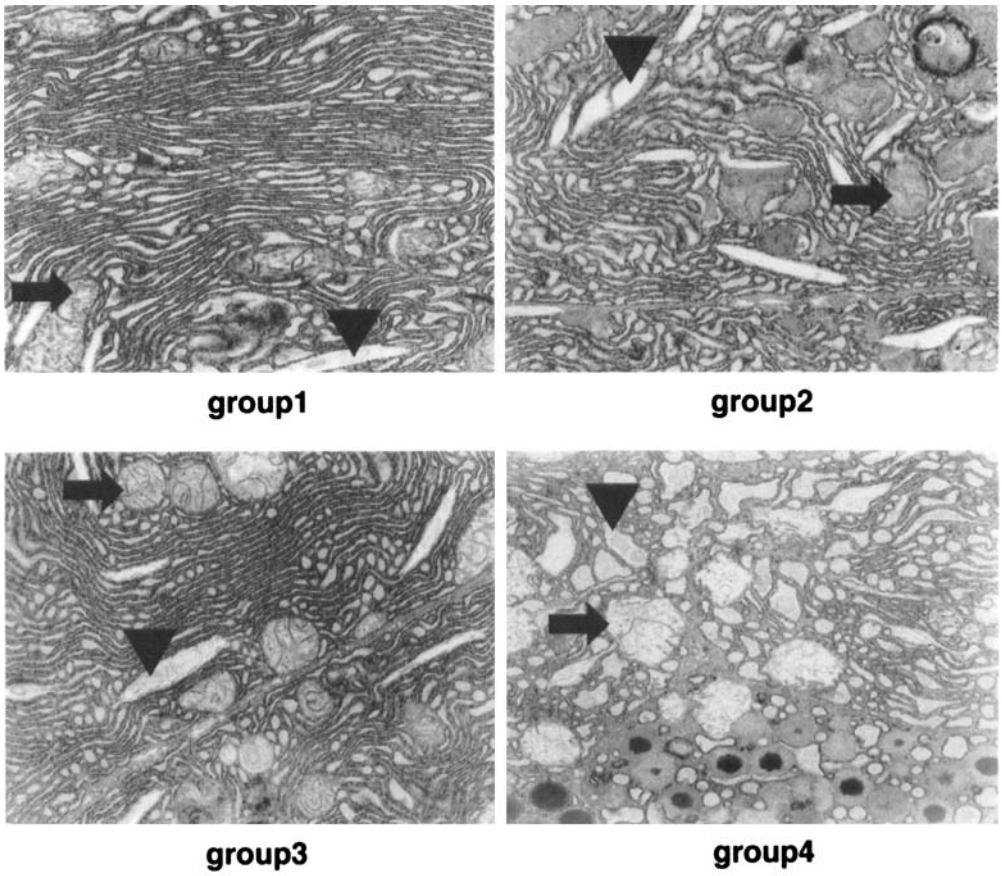

Fig. 1. Electron micrographs showing acinar cells after preservation in groups 1, 2, 3, and 4. Vacuolar RER (*arrow head*) was observed to be more intense in groups 2 and 4 than in groups 1 and 3. Mitochondrial swelling (*arrow*) was slightly more prominent in groups 2 and 4 than in groups 1 and 3. (original magnification × 8,000)

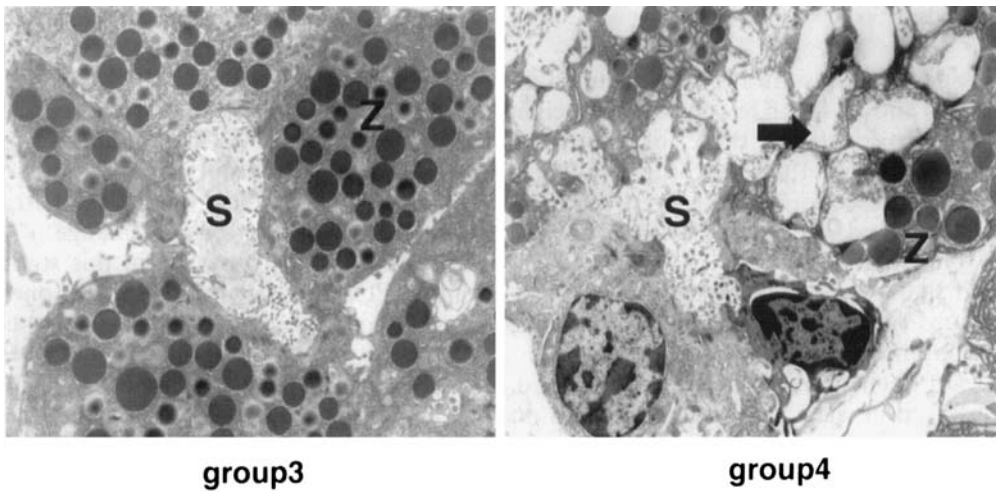

Fig. 2. Electron micrographs showing acinar cells and centroacinar cells surrounding a secretory capillary (*S*) in groups 3 and 4. Zymogen granules (*Z*) in group 3 showed no remarkable changes. In contrast, degenerative changes in zymogen granules (*arrow*) are evident in group 4, particularly in the area close to the luminal surface

and 3 compared to UW groups 2 and 4 ($P < 0.01$). No significant difference was observed between group 1 and the control in this RER analysis either (Fig. 4).

Discussion

The current standard of organ preservation is simple cold (4°C) storage in University of Wisconsin solution (UWM). By this method, canine pancreatic grafts can be preserved for up to 72h; the high osmotic forces of this solution prevent cell swelling [18]. However, this method does not provide significant molecular oxygen support for maintaining tissue oxidative processes. Even when using simple storage in UW with oxygen bubbling, the pancreas is not oxygenated, and consequently ATP is not resynthesized [10]. To reduce the ischemic injury of pancreatic grafts during simple cold storage, we developed TLM [7] that continuously supplied sufficient oxygen to the pancreas through the preservation period [8, 10]. Subsequently, we demonstrated that oxygenation of the pancreas leads to intragraft ATP production during preservation [9] and that this method maintains pancreatic graft viability for up to 96h in the canine model [2, 6]. This is the first study to analyze

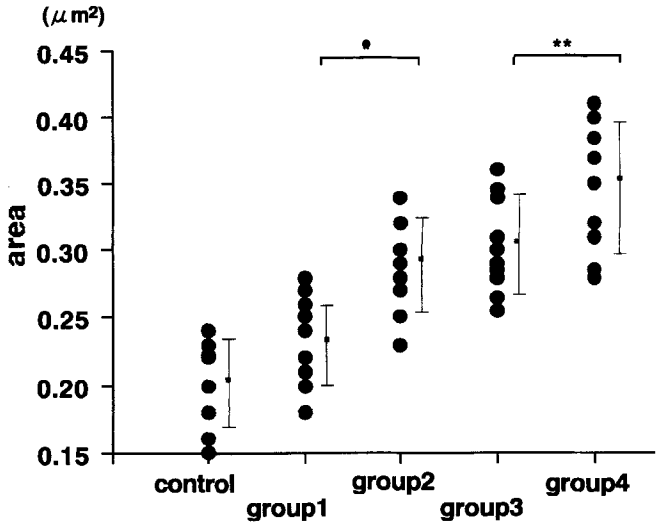

Fig. 3. The mean area of one mitochondrion analyzed by computer-graphic analyses using NIH image 1.62f soft. Mitochondrial swelling significantly increased depending on preservation time and manner. In addition, it increased significantly in group 2 compared with that of group 1, and in group 4 compared with that of group 3. It is notable that no significant difference was observed between group 1 and the controls. *A closed circle* indicates the mean value of 20 fields in one dog. $*P < 0.01$; $**P < 0.05$

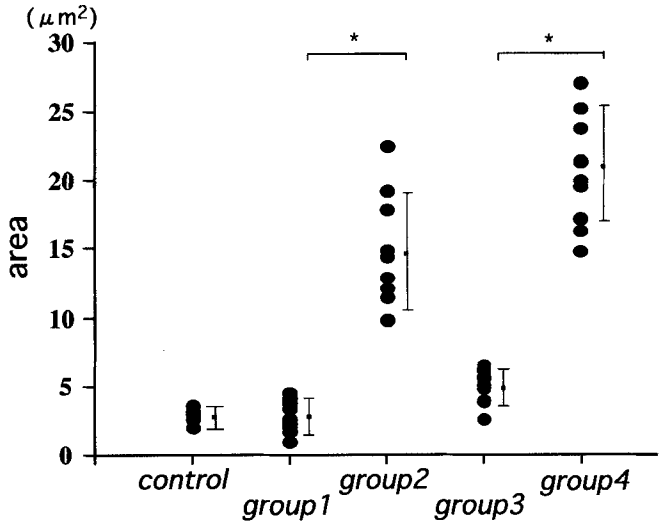

Fig. 4. The mean area of vacuolar RER in one field analyzed by computer-graphic analyses using NIH image 1.62f soft. Vacuolization of RER was significantly less in groups 1 and 3 than in groups 2 and 4. Note that no significant difference was observed between group 1 and the controls. *A closed circle* indicates the mean value of 20 fields in one dog. $*P < 0.01$

ultrastructurally canine pancreatic grafts preserved by these two preservation techniques.

By light microscopy, pancreatic structure, cell size and shape were well preserved by both methods. No morphological alteration was detected in the light microscopic analysis. In contrast, electron microscopic analyses clearly demonstrated mitochondorial swelling and RER vacuolization in acinar cells to various extents, depending on preservation method and -time. These ultrastructural alterations have been reported as early responses of graft cells to stress in hepatocytes and small intestinal cells, and they were not visible with light microscopic analysis [1, 3, 4, 11, 12, 14]. In this study, the same ultrastructual alterations were detected in pancreatic acinar cells, they were also visible at an earlier stage than with light microscopy. Thus, pretransplant graft assessment by electron microscopy may prove to be a useful tool for predicting posttransplant outcomes. However, influences of these ultrastructual alterations on post-reperfusion graft viability require further investigation on the correlation with ischemia/reperfusion injury including cellular apoptosis and lipid peroxydation reaction.

Our ultrastructural analyses showed that morphological changes, in particular RER vacuolization, were significantly less in the TLM groups than in the UWM groups. Moreover, it is notable that no significant differences in mitochondrial- and RER alteration were observed in ultrastructural morphometric analyses between group 1 (TLM for 72h) and the control. These results confirmed the impact of oxygen delivery during preservation on the maintenance of graft ultrastructural morphology. However, since mitochondrial swelling increased during preservation in a time-dependent manner by each method, mitochondria may be strongly influenced not only by hypoxia but also by hypothermic stress as was seen in the liver model [13].

Cytosolic free calcium concentration is modulated by membrane-associated, ATP-dependent Ca^{2+}, Mg^{2+}-ATPases. ATP depletion due to ischemia causes an early increase in cytosolic calcium concentration. Increased Ca^{2+} in turn activates a number of enzymes with potential deleterious cellular effects, such as phospholipases, proteases, ATPases, and endonucleases [16, 17]. Our results suggest that continued intragraft ATP synthesis by TLM may prevent the intracellular deleterious chain of events during cold ischemia, which maintains the integrity of intracellular organelle membranes and cellular membranes, although intracellular Ca^{2+} and protease levels were not determined in this study.

Degenerative changes of zymogen granules in the areas close to the luminal surface were evident only in the UWM groups. These changes were not seen in the TLM groups. This implies that zymogen granule membrane integrity may also be well maintained by TLM and that some auto-activated trypsinogen from degenerative zymogen granules in UWM groups may result in graft duct and parenchymal damage during preservation. Since this is only a speculation from the morphological findings, we need further biochemical analyses of this issue.

In conclusion, through ultrastructural analyses, TLM demonstrated enhanced protection of intracellular organelles, mitochondria and RER, up to 72–96h. Well-maintained graft ATP in TLM groups may be responsible for maintaining the integrity of intracellular organelle membranes as well as cellular membranes. In addition, ultrastructural analyses prior to reperfusion may be a useful tool for predicting posttransplant graft outcomes.

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