

# Preservation of Pancreatic Tissue Morphology, Viability and Energy Metabolism during Extended Cold Storage in Two-Layer Oxygenated University of Wisconsin/Perfluorocarbon Solution

Shahar Atias<sup>1\*</sup>, Solly S. Mizrahi MD FACS<sup>2</sup>, Ruthy Shaco-Levy MD and Alexander Yussim MD

<sup>1</sup>Faculty of Health Sciences, Ben-Gurion University of the Negev, and Departments of <sup>2</sup>Surgery A and <sup>3</sup>Pathology, Soroka Medical Center and Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel

<sup>2</sup>Department of Transplantation, Rabin Medical Center (Beilinson Campus), Petah Tikva, Israel

**Key words:** perfluorocarbon, University of Wisconsin solution, pancreatic cells, two-layer method, preservation of pancreas

## Abstract

**Background:** In contrast to the relative scarcity of donor kidneys and hearts, the potential supply of deceased donor pancreata is exceeding the demand. However, this organ surplus is not being fully realized because, in current transplantation practice, the duration of pancreas storage before transplantation is limited to 8–10 hours due to the extreme vulnerability of pancreatic tissue to anaerobic damage caused by preservation.

**Objectives:** To reduce cold ischemic injury in order to increase the utilization of donor pancreases in Israel for whole-organ and cell transplantation.

**Methods:** We evaluated a novel two-layer preservation oxygenated cold storage method that uses perfluorocarbon to continuously supply oxygen to the pancreas during preservation in conventional University of Wisconsin solution.

**Results:** Pancreatic tissue morphology, viability and adenosine-triphosphate content were serially examined during preservation of the pig pancreas for 24 hours either by a two-layer or by conventional simple cold storage. Already after 12 hours of storage, the superiority of the two-layer method over the University of Wisconsin method was apparent. Starting at this time point and continuing throughout the 24 hours of preservation, the tissue architecture, mitochondrial integrity, cellular viability and ATP tissue concentration were improved in samples preserved in oxygenated UW/PFC as compared to controls stored in conventional UW solution alone.

**Conclusions:** The UW/PFC two-layer preservation method allowed tissue ATP synthesis and amelioration of cold ischemic tissue damage during extended 24 hour pancreas preservation. This method could be implemented in clinical practice to maximize utilization of pancreata for whole-organ and islet transplantation as well as for pancreas sharing with remote centers.

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Although 50%–60% of cadaveric donors are acceptable for multi-organ donation, only 20–25% of them are currently used for pancreas transplantation. In Israel, the situation is especially worrisome, considering that in the last decade (1996–2006) only 5–11 pancreases versus 22–49 livers were transplanted annually

[1]. Analysis of large international databases shows that the major cause of under-utilization of donor pancreata is the increased susceptibility of pancreatic tissue to impaired hemodynamics of the donor (warm ischemia) and to the duration of cold preservation [2].

A technique known as the two-layer method, which has been tested in several centers in Japan, the United States and Europe, has shown an advantage over the currently used method that uses cooled University of Wisconsin solution for preservation [3–6]. In addition to the improved viability and maintenance of structural integrity of whole pancreas, the two-layer method was found to increase the number and quality of active islet cells that are isolated from the pancreas after preservation [7]. These results suggest that TLM may help to increase the number of successful whole pancreas and islet transplantations [4–6]. The safe extension of preservation time could be especially useful in countries, like Israel, planning to initiate an islet transplantation program in collaboration with an established islet isolation facility located in another country [8].

Using an animal model, the present study tested the feasibility and logistics of the two-layer method in pancreas preservation with the aim of implementing this modality in the clinic to increase the utilization of donor pancreases in Israel for whole-organ and cell transplantation.

## Materials and Methods

A 2 month old female pig of mixed type (large white/Landrace) weighing 20 kg was purchased from the Institute of Animal Research, Kibbutz Lahav, Israel. The following solutions were used for pancreas preservation: standard preservation University of Wisconsin solution (NPBI International NL) in the control group, and TLM solution, consisting of UW and perfluorocarbon (F2 Chemicals Ltd UK) in the study group, charged for 2 hours with oxygen supplied from conventional bottles equipped with a bubble filter allowing flow of 5 L/min.

## Operative procedure

The operative procedure was approved by the Institutional Review Board for Animal Experimentation of Soroka Medical Center and conducted in accordance with International Guidelines for Animal Experiments [9].

\* This study fulfilled part of the requirements for the MD degree of the first author

ATP = adenosine-triphosphate

UW = University of Wisconsin

PFC = perfluorocarbon

Intraoperative anesthesia consisted of induction with an intramuscular injection of 20 mg/kg ketamine hydrochloride (Fort Dodge Animal Health, Inc, Indiana, USA) and 2 mg/kg xylazine (V.M.D., Belgium), followed by a mask of 1% isoflurane (Minrad Inc., USA) inhalation, until intubation, and maintained with 1–3% isoflurane throughout the operation.

Laparotomy was performed through a midline xyphopubic incision, and the abdominal aorta and splenic vein were cannulated and perfused with a saline solution at 4°C. The animal was exsanguinated through the incision in the vena cava. After cooling the pancreas, achieved by replacing the blood with the cold saline and packing the area around the pancreas with sterile ice, the entire pancreas was removed. The animal expired from cardiac arrest shortly after exsanguination.

### Preservation

For the standard UW preservation (control method) a sterile bag was filled with UW solution and cooled to 4°C by ice in a polystyrene box. For TLM (study method) a special container was filled with 500 ml PFC solution and with the same amount of UW solution. Since PFC is heavier than UW solution, two layers of solutions were placed in the container, with the PFC at the bottom. Oxygen was filtered through the PFC for 2 hours before the preservation and the container was cooled to 4°C.

Following harvest, the pancreas was cut longitudinally into two equal parts, and one was placed in the control (UW) and the other in the study TLM (oxygenated UW/PFC) solution.

### Study design

From each pancreatic half preserved by conventional (UW) or two-layer method, equally sized tissue samples were removed from the respective solutions at baseline and after 8, 12, 16 and 24 hours of preservation. At these time points, each sample was tested for morphology by light and electron microscopy, tissue viability by nicotinamide adenine dinucleotide staining, and for tissue energy reserves by determination of adenosine-triphosphate tissue levels.

### Methods

For light microscopy, each sample was fixed in 10% formalin, processed and embedded in paraffin. Sections of 5  $\mu$  thick were stained with hematoxylin and eosin. For electron microscopy, the tissue samples were placed immediately after extraction in fixative (Karnofsky solution), washed with cacodilate buffer 0.1 M pH 7.2, post-fixed with osmium tetroxide 1% (cacodilate buffer), dehydrated with ethanol and, finally stained with uranyl acetate and lead citrate.

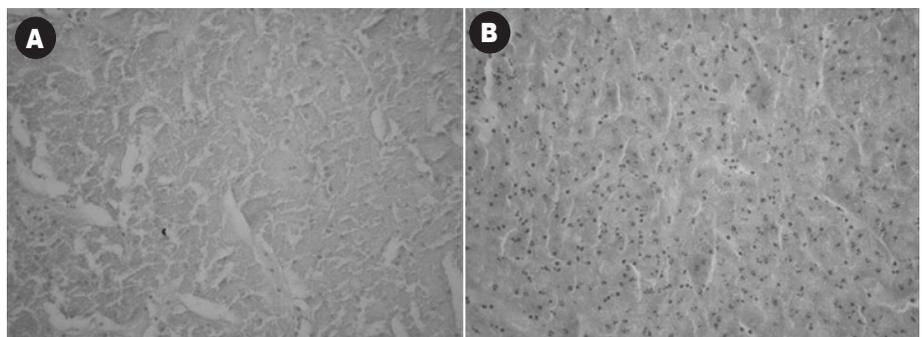
The ultrastructure of the specimens

was examined, including the appearance of the nuclei, rough endoplasmic reticulum, mitochondria and Golgi apparatus. For NADH viability staining, each sample was frozen in liquid nitrogen-cooled isopentane, and kept at -80°C. Cryostatic sections and enzyme physicochemical preparations were made from the frozen samples for detection of NADH diaphorase. The NADH procedure was conducted according to the protocol as described [10].

For ATP concentration measurement, each sample was rapidly frozen in liquid nitrogen, and kept at -80°C for further evaluation. The tissue was grinded and put into 3 ml of ice cold perchloric acid 10%. The proteins were removed by centrifugation for 10 minutes at 4500 rpm. Then, 0.5 ml of the supernatant was neutralized by adding 0.2 ml of KOH, and then by adding TRIS acetate 0.1 M/EDTA 2 mM (pH 7.7). The buffered solution was centrifuged for 5 min at 4500 rpm. Subsequently, 0.05 ml of the supernatant was mixed with 0.05 ml of CellTiter-Glo™ reagent containing luciferin, then luciferase was added and the solution was placed on plates. The plates were mixed on an orbital shaker for 2 min to induce cell lysis. In the presence of ATP, Mg<sup>2+</sup> and molecular oxygen, mono-oxidation of luciferin was catalyzed by luciferase, generating a luminescence signal. The plate was then incubated for 10 min at room temperature to stabilize the luminescence signal. Luminescence was recorded using the Kodak Digital Science Image Station. The results were analyzed by TINA software that determined ATP concentration by measuring the intensity of colored wells in the assay plate (Promega, Madison, WI, USA) [11].

### Results

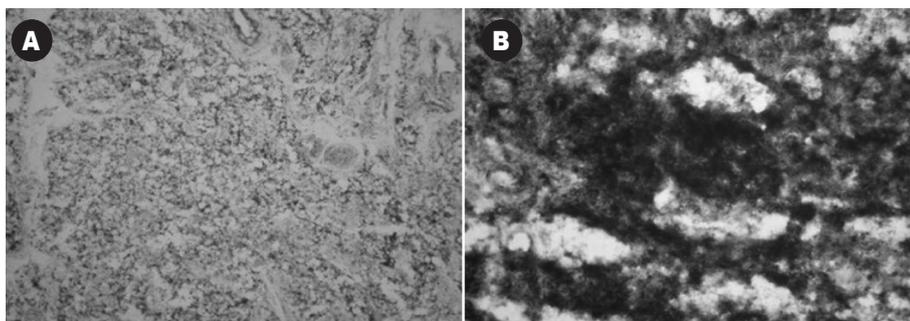
On light microscopic examination, after only 8 hours of preservation the tissue preserved in the UW solution started to show signs of necrosis, including reduction in cell size, nuclear pyknosis, and loss of normal tissue structure with amorphous areas, especially at the periphery. After 24 hours, the extent of damage in the tissue preserved in the UW solution had spread to > 95% of the sample. In contrast, in the tissue preserved with TLM the damage was less apparent, and after 24 hours involved < 60% of the section [Figure 1].



**Figure 1. [A]** Pancreatic tissue after 12 hours of preservation in UW solution, showing extensive necrosis. Note loss of nuclei and presence of "ghost cells" (hematoxylin & eosin stain, original magnification x 400).

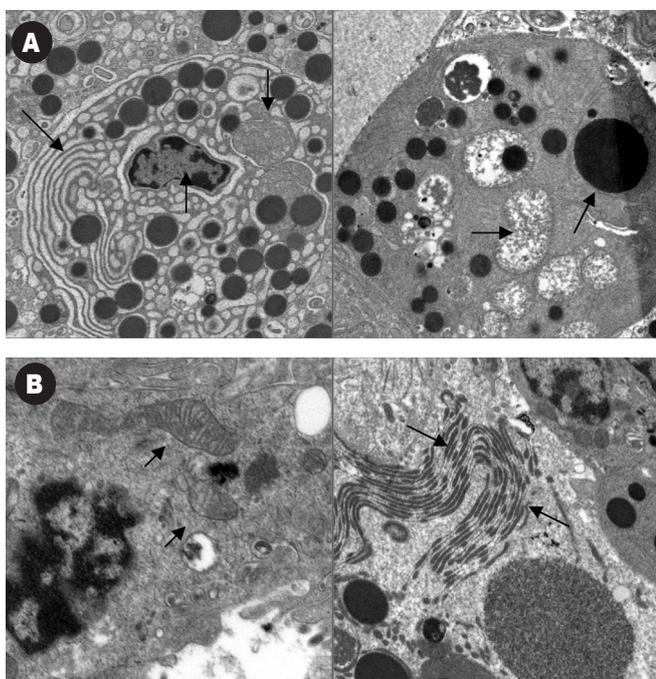
**Figure 1. [B]** Pancreatic tissue after 12 hours of preservation in TLM solution, showing preserved architecture. Note the preserved nuclei and well-demarcated cellular borders (H&E stain, original magnification x 400).

NADH = nicotinamide adenine dinucleotide



**Figure 2. [A]** Non-viable tissue after 24 hours in UW solution. Note the weak stain of the tissue, indicating that it is no longer viable and cannot capture the color (NADH-dephorase stain, original magnification x 400).

**Figure 2. [B]** Viable tissue (stains blue) after 24 hours of preservation in UW/PFC solution. Note the intensity of the stain, which indicates the viability of the cells capturing the color (NADH-diaphorase stain, original magnification x 400).



**Figure 3. [A]** Electron micrographs showing necrotic cells containing broken and swollen mitochondria, pyknotic nuclei and vacuolar rough endoplasmic reticulum (arrows) after 16 hours of preservation in UW solution (original magnification x 1500). **[B]** Electron micrograph showing viable cells containing undamaged mitochondria and golgi apparatus (arrows) after 16 hours of preservation in UW/PFC solution (original magnification x 2500)

NADH staining also showed a distinct difference in tissue viability of samples preserved in conventional UW and TLM solutions. The percentage of viable tissue after 24 hours of preservation was higher under TLM preservation than under UW preservation: > 40% versus < 5% respectively [Figure 2].

Electron microscopy measured parameters such as mitochondrial damage, size of nuclei, presence of cisterns in the rough endoplasmic reticulum, amount of cytoplasm and size of the cells, amount of apoptotic cells, and areas of necrosis. After 8 hours of preservation, broken and swollen mitochondria were seen in the tissue preserved in UW, after 12 hours they were

identified in larger areas, and after 16 hours they occupied nearly the entire surface of the tissue section. In contrast, in samples preserved by TLM, after 12 hours of preservation only shrinkage of cells was observed. After 16 hours, swollen mitochondria were focally identified, but even after 24 hours a large amount of unaffected or slightly damaged tissue was still observed [Figure 3].

The most distinct difference between the two preservation methods was demonstrated by tissue ATP concentration measurement. After 16 hours the ATP concentration in the sample preserved in UW solution had decreased to 22% of

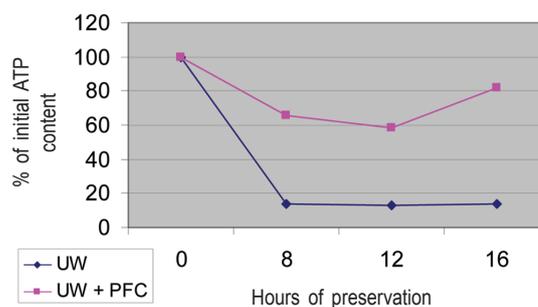
its initial level, while the ATP concentration in the corresponding sample preserved in TLM maintained 82% of its initial level [Figure 4].

In summary, 12 hours after beginning the preservation, the parameters examined – namely, tissue integrity, viability and energy metabolic reserves – showed that the two-layer preservation method was superior to conventional UW preservation.

## Discussion

The current method of preserving donor pancreata is simple cold storage in UW solution. In this study, on the ability of the two-layer method to reduce cold ischemic injury in preserved pancreas, analysis of tissue morphology and metabolic energy reserves demonstrated that the TLM solution significantly reduced the detrimental changes in the pancreatic tissue preserved up to 24 hours as compared to the conventional UW cold storage method. The plausible explanation is that TLM, by supplying oxygen for tissue metabolism, enhances tissue ATP synthesis, which in turn ameliorates tissue damage during extended anaerobic preservation [12].

A recent study reported similar results in experimental pancreas preservation with oxygenated one-layer PFC alone [13]. Interestingly, in his later publications, the same author (D. Brandhorst) concluded that pancreas storage in oxygenated PFC did not restore post-transplant function of isolated pig islets pre-



**Figure 4.** Measured ATP during 16 hours of preservation in both preservation methods tested. As seen in the graph, the levels of ATP were significantly higher in the UW/PFC solution than in the UW solution throughout the preservation time.

damaged by warm ischemia and even questioned the value of oxygenated preservation in islet isolation and transplantation in general [14,15]. On the other hand, the same author points out significant differences in study design between his and several other reports confirming TLM utility. In contrast to other prospective studies, coming mostly from the U.S., which had used continuous oxygenation, shorter periods of preservation and younger donors, Brandhorst retrospectively analyzed a large European database of older donor pancreata preserved over prolonged periods with static (one time charge) oxygenation. In our view, his report does not invalidate the significance of TLM, but by pointing out the differences in methodology of tissue oxygenation, organ selection and the duration of preservation used by different centers, in fact encourages further investigation of conditions for optimal application of this method. Despite this controversy, the two-layer method has already found its way into the clinic and has been successfully used in numerous transplant centers for islet isolation and transplantation of the islets and whole pancreata, including those harvested in remote centers and shipped over long distances [4-8].

In conclusion, the UW/PFC two-layer preservation method allows tissue ATP synthesis and amelioration of cold ischemic tissue damage during extended 24 hour pancreas preservation. This logistically simple method could be adapted easily to the clinical setting and used to maximize utilization of pancreata for whole-organ and islet transplantation as well as for pancreas sharing with remote centers, although further investigations of conditions for optimal application of this method are warranted.

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**Correspondence:** Shahar Atias, 46 Asa Street, Kiryat Gat 82067, Israel.

Phone: (972-50) 686-0601

Fax: (972-8) 640-3260

email: atiassh@bgu.ac.il