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ORIGINAL ARTICLE

Intermittent capillary perfusion in rat pancreas grafts following short- and long-term preservation in University of Wisconsin solution

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Summary

In pancreas transplantation (PTx), ischemia/reperfusion-induced deterioration of graft-microcirculation is accompanied by alterations of intermittent capillary perfusion (IP; alternating cessation and resumption of capillary blood flow) is known to counteract malperfusion. Incidence and effectiveness of IP following short- versus long-term preservation of pancreas grafts with University of Wisconsin (UW) solution has not been examined so far. PTx was performed in Lewis rats following 2-h or 18-h preservation in UW solution. Using intravital fluorescence microscopy, functional capillary density (FCD), red blood cell (RBC) velocity, IP-incidence and -frequency were analyzed. Laser Doppler flowmetry allowed for the determination of erythrocyte flux and velocity. Measurements were performed at 30, 60 and 120 min after reperfusion. Non-transplanted animals served as controls. FCD, RBC-velocity and -flux remained unchanged in the 2-h group. IP was encountered in 87% of all observation areas at 120 min. After 18-h ischemia, FCD was significantly reduced, which was paralleled by a 50% incidence of IP at 120 min. Tissue edema and leukocyte infiltration in pancreas grafts following 18-h preservation were significantly enhanced. Therefore, IP is an important mechanism aimed at improving microcirculation and UW solution is suitable to preserve vasomotion-activities enabling long-term preservation in a pancreas graft.

Introduction

Graft pancreatitis and thrombosis remain major problems in clinical pancreas transplantation causing considerable morbidity and graft loss [1]. The rate of early technical graft loss ranges between 6.6% and 10%, with an incidence for graft thrombosis alone of up to 7.8% [2]. Graft pancreatitis is observed in up to 20% of all patients [3]. Especially increased preservation time seems to be a risk factor for these complications [4], which are related to the ischemia/reperfusion (IR)-injury [1,5]. In the pancreatic microcirculation vasomotion-activities have been identified to be responsible for the regulation of tissue perfusion and finally also for the extent of the IR-injury. Arteriolar vasomotion indicates a rhythmical change between vasoconstriction and -dilation, inducing flow motion, a synchronous change of red blood cell (RBC) velocity in arterioles and downstream capillaries and intermittent capillary perfusion (IP), an alternating complete cessation and resumption of capillary blood flow [6]. Under conditions of malperfusion particularly IP is thought to improve oxygen transport to critically

perfused tissues [7,8]. In the transplanted pancreas a high incidence of IP, which was paralleled by preserved microvascular perfusion, could be demonstrated following short-term preservation for 1 h in histidine–tryptophane–ketoglutarate (HTK) solution [9]. However, after prolongation up to 6 h, IP almost completely disappeared and microvascular perfusion of the pancreas grafts worsened markedly in this study.

Although IP seems to improve graft microcirculation, factors influencing the incidence and quality of IP following pancreas transplantation are poorly understood. Besides sympathetic denervation during the procedure of organ harvest, the duration of the warm and cold ischemic intervals and the choice of the optimal preservation solution, with different electrolyte and colloid compositions, could be of particular relevance to induce IP and thus to maintain adequate microvascular tissue perfusion. University of Wisconsin (UW) solution has been used widely for the preservation of pancreas grafts and proved its safety and efficacy in a number of clinical and experimental investigations [10–12]. However, if UW solution is suitable for preservation of vasomotion-activities like IP, which might be a decisive factor to enable long-term preservation, is unknown up to now. Therefore, we assessed IP in the early reperfusion period of the transplanted rat pancreas following short- (2 h) and long-term (18 h) preservation with UW solution. Furthermore, we compared the microcirculatory analysis of intravital fluorescence microscopy (IVM) with measurements obtained by laser Doppler flowmetry (LDF), a method for clinical assessment of microcirculation.

Materials and methods

All experiments were approved by the local government and carried out at the Institute for Surgical Research at Munich University in accordance with the 'Principles of Laboratory animal care' of the National Institutes of Health publication No. 85–23, revised 1985.

Anesthesia

Inbred male Lewis rats ($n = 37$) weighing 201 ± 4 g were used in this study. After overnight fasting but free access to tap water animals were anesthetized by chloralhydrate (360 mg/kg i.p.), tracheotomized and mechanically ventilated with an oxygen/nitrous-oxide mixture (ventilatory rate 50–60 min^{-1}, tidal volume 10 ml/kg, inspiration/expiration ratio 1:1, airway pressure <5 mmHg, $FiO_2 = 0.3$–0.35; Harvard Rodent Ventilator 683, Harvard Apparatus, Holliston, MA, USA) for maintenance of normal arterial blood gases ($paO_2 = 100$–130 mmHg, $paCO_2 = 35$–40 mmHg). Adequate anesthesia was achieved throughout the experiments by intravenous infusion of α-chloralose (20 mg/kg/h). Left carotid artery and jugular vein were cannulated with polypropylene catheters for continuous recording of mean arterial blood pressure (MABP) and fluid replacement (4 ml/h NaCl i.v.) as well as for injection of the fluorescent compounds in the recipients. Body temperature was maintained between 36 and 37 °C by placing the animals in supine position on a heating pad.

Surgical procedure

Harvesting of the pancreaticoduodenal graft was performed according to a modified technique described by Lee *et al.* in detail [13]. Briefly, pancreas and duodenum were mobilized at an aortal segment including coeliac trunc and superior mesenteric artery. Following heparinization (200 IE i.v.) the aorta was cannulated, the portal vein cut at the bifurcation and the pancreas flushed with 10 ml chilled UW solution (pressure: 50 cm H_2O; Belzer UW-CSS, Du Pont Pharmaceuticals, Wilmington, DE, USA). Finally, after the dissection of the aortal segment, the graft was removed from the donor and stored for either 2 or 18 h in 4 °C cold UW solution.

In the recipient animal the left kidney was removed and the pancreaticoduodenal graft anastomosed to the renal vessels using a nonsuturing cuff technique [14]. After simultaneous release of the clamps, the pancreaticoduodenal graft was carefully exteriorized on a specially designed stage for IVM. The pancreatic surface was covered with Saran wrap and superfused with warm, bicarbonate-buffered Ringer's solution. Exocrine secretions were allowed to flow off via a catheter placed in the distal part of the duodenum.

Experimental protocol

Recipient animals of the short-term preservation group received pancreas grafts following 2-h ischemia (2 h-I; $n = 8$) in cold UW solution, whereas animals of the long-term preservation group received pancreas grafts after 18-h hypothermic ischemia (18 h-I; $n = 7$) stored in the same solution. Seven nontransplanted animals with exteriorization of the pancreas served as controls (Con). When the recipient animals met the entry criteria (MABP 80–120 mmHg; HR >350/min, $paO_2 = 100$–130 mmHg; $paCO_2 = 30$–40 mmHg), revascularization and reperfusion were commenced. Warm ischemic intervals during revascularization of the grafts did not differ significantly between the 2 h-I and 18 h-I group with 10.5 ± 1.0 and 11.0 ± 1.5 min, respectively. Macrohemodynamic parameters were continuously monitored. Microcirculatory analyses were performed 30, 60 and 120 min after onset of reperfusion. All animals survived the 120-min reperfusion period.

Intravital fluorescence microscopy and laser Doppler flowmetry

Intravenous injection of 0.1 ml fluorescein isothiocyanate (FITC)-labeled hydroxyethylstarch (0.75%; MW 200000/0.6; Laevosan, Linz, Austria) allowed for contrast enhancement of microvessels for IVM. A modified Leitz-Orthoplan microscope (Leitz, Wetzlar, Germany) with a 100 W HBO lamp, attached to a Ploemo-Pak illuminator with $I_{2/3}$ (excitation 450–490 nm/emission >515 nm) filter block was used for epi-illumination. A water immersion objective (×25/0.6, Leitz) facilitated a final magnification of 820× on the video screen (PVM 2042 QM, Sony, Germany). The observations were recorded by means of a charge-coupled device video camera (FK 6990, COHU, Prospective Measurements, San Diego, CA, USA) and transferred to a S-VHS video recorder (AG-7330, Panasonic, Tokyo, Japan) for offline evaluation. The recording time was 60 s for each observation field. For LDF an optical probe was placed on the surface of the pancreas graft, avoiding pressure application to the tissue by means of a micromanipulator. The LDF signal was continuously computed during the 60-s observation period (Laser Blood Flow Monitor MBF3D, Moor Instruments Ltd, Millwey/Axminster, UK).

Microcirculatory analysis

Quantitative analysis of the microcirculation of pancreatic acinar tissue included the offline determination of functional capillary density (FCD), capillary RBC velocity and IP. During IVM, a total of seven regions of interest (ROI; 400 μm × 350 μm) were chosen in a standardized way: in the head of the pancreas graft three ROIs were selected, in pancreatic body and in tail two ROIs. FCD, which is defined as the length of all blood-cell-perfused capillaries (cm) per observation area (cm^2), was assessed with a stereological method [15]: a defined grid system was superimposed on the video screen. Counting the intersections between grid lines and RBC-perfused capillaries (N_c) allowed for calculation of the FCD according to the equation

$$\mathrm{FCD} = \frac{\pi N_c}{2\,L}[\mathrm{cm}^{-1}],$$

where L is the total length of the grid lines. RBC velocity (mm/s) was measured by frame-to-frame analysis using a computer-assisted image analysis system (CAMAS, Zeintl Software Engineering, Heidelberg, Germany). IP was defined as alternating cessation (RBC velocity = 0 mm/s) and resumption (RBC velocity > 0 mm/s) of capillary perfusion. Quantitative assessment of IP included the determination of ROIs presenting with IP (%), its frequency in cycles per minute (cpm) and the flow period per cycle (%). With LDF erythrocyte flux and velocity in the microcirculation of the ∼1 mm^3 sample volume at seven ROIs were recorded. The LDF data represent relative perfusion values and the results are thus expressed in arbitrary units (AU). The biological zero of rat pancreas, representing background activity in the Doppler signal, was taken into account [16].

Histology

At the end of the experiments, pancreatic tissue was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with a chloroacetate esterase technique for light microscopic analysis. Leukocyte infiltration into tissue was analyzed histomorphologically by counting extravasated chloroacetate esterase-stained leukocytes in 60 high-power fields (HPF; n/mm^2) per animal. Interstitial and intralobular edema formation was assessed by a planimetric area analysis (percentage edema area of total tissue area) also in 60 HPF.

Statistics

Results are expressed as means ± standard error of the mean (SEM). Kruskal Wallis one-way analysis of variance on ranks followed by Dunn's test were applied for the assessment of different groups. For time interactions Friedman repeated measures ANOVA on ranks followed by Dunn's test were used. Differences were considered significant at a $P < 0.05$ level.

Results

Hemodynamics

Mean arterial blood pressure remained stable throughout the reperfusion period without group or time interaction.

Functional capillary density and RBC velocity

In the 2 h-I group nutritive perfusion in exocrine tissue was not altered when compared with controls: FCD was 618 ± 6, 591 ± 7, 574 ± 8 cm^{-1} after 30, 60 and 120 min. After 120 min FCD was significantly reduced when compared with 30 min of reperfusion.

In the 18 h-I group FCD was reduced significantly to 462 ± 5, 421 ± 10 and 421 ± 8 cm^{-1} after 30, 60 and 120 min of reperfusion, respectively, when compared with controls and the 2 h-I group. FCD significantly declined after 60 and 120 min when compared with 30 min of reperfusion. RBC velocity (0.57–0.59 mm/s) remained unchanged when compared with controls (Fig. 1).

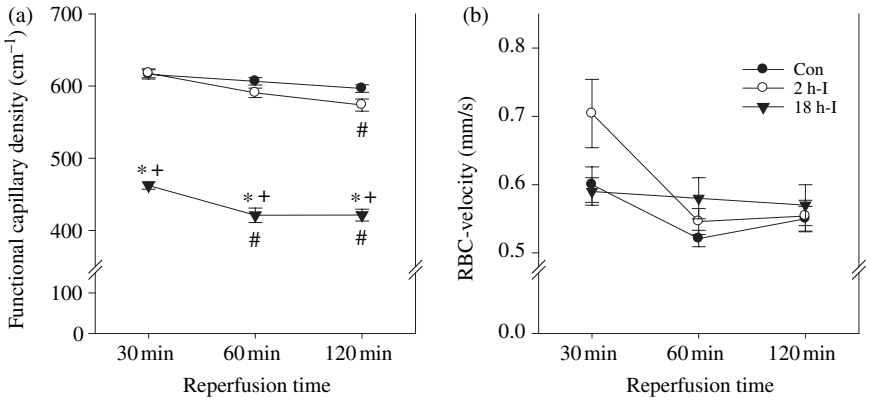

Figure 1 (a) Functional capillary density (cm^{-1}) and (b) red blood cell (RBC) velocity (mm/s) in exocrine tissue of pancreas grafts examined by intravital fluorescence microscopy following either 2 h (○) or 18 h (▼) hypothermic preservation in University of Wisconsin solution and 30, 60 and 120 min of reperfusion. Nontransplanted animals served as controls (●). Data shown represent mean ± SEM; *P < 0.05 versus controls; +P < 0.05 versus 2-h ischemia group; #P < 0.05 versus 30 min of reperfusion.

Intermittent capillary perfusion

In the 2 h-I group IP was absent at 30-min reperfusion. Instead, a continuous capillary blood flow, with high RBC velocity (0.70 ± 0.05 mm/s; Fig. 1b), was observed. After 60 min of reperfusion IP presented in 45 ± 15% and after 120 min of reperfusion in 87 ± 3% of the ROIs (Fig. 2a). Quantitative analysis of the IP-dynamics in this group revealed a frequency of 4.2 ± 0.2 cpm with a mean flow period per cycle of 47 ± 5% at 60 min of reperfusion and a frequency of 4.1 ± 0.1 cpm with a mean flow period per cycle of 46 ± 1% after 120 min of reperfusion (Fig. 2b and Table 1). In the control group, IP was absent throughout the whole experimental period.

Also in the 18 h-I group, IP was observed. After 30 min of reperfusion 2 ± 2%, after 60 min of reperfusion 23 ± 10% and after 120 min of reperfusion 50 ± 15% of all ROIs showed IP, significantly less than in the 2 h-I group. Frequency of the IP cycles was 4.3 ± 0.2, 3.9 ± 0.1 and 3.2 ± 0.3 cpm (Fig. 2) with a flow fraction per cycle of 51 ± 1%, 53 ± 6% and 54 ± 4% after 30, 60 and 120 min of reperfusion, respectively, not significantly different from the frequency spectrum of the 2 h-I group after 60 and 120 min of reperfusion (Table 1).

Laser Doppler flowmetry

Flux values in the 2 h-I group obtained by LDF indicated preserved microvascular perfusion when compared with the controls. Flux was 253 ± 23, 206 ± 20 and 251 ± 15 AU in group 2 h-I versus 264 ± 15, 240 ± 13 and 238 ± 7 AU in the control group at the three time points of measurement, respectively (Fig. 3a). The analysis of RBC velocity by LDF in the 2 h-I group neither did reveal the initial increase of the RBC velocity at 30 min of reperfusion nor was a significant signal reduction or periodical fluctuation after 60 and 120 min of reperfusion detected, as would be expected with the begin of IP (Fig. 3b).

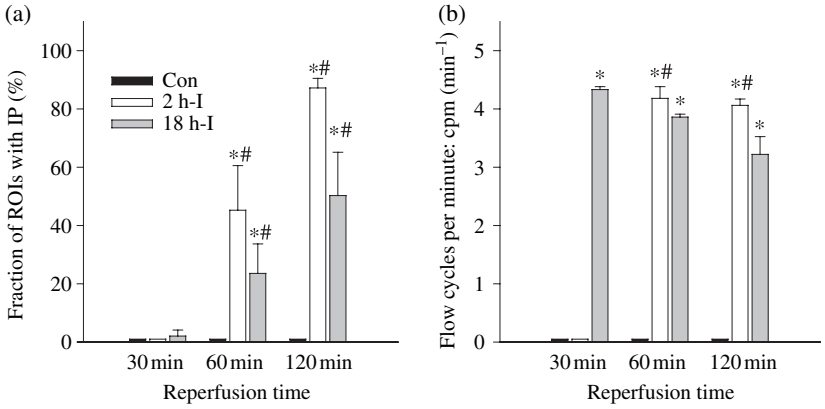

Figure 2 (a) Fraction of the ROIs (%) presenting with intermittent capillary perfusion (IP) and (b) its frequency (min^{-1}) in exocrine tissue of pancreas grafts during 2 h of reperfusion following either 2-h (white bars) or 18-h (grey bars) hypothermic ischemia. Nontransplanted control animals did not show IP in the native pancreas (black bars). Data shown represent mean ± SEM; *P < 0.05 versus controls; +P< 0.05 versus 2 h ischemia group; #P < 0.05 versus 30 min of reperfusion.

Table 1. Fraction of flow time (%) in a cycle of stop-and-go during intermittent capillary perfusion (IP) in pancreas grafts following different periods of hypothermic preservation in University of Wisconsin solution and 2 h of reperfusion.

	30-min reperfusion	60-min reperfusion	120-min reperfusion
Control	100	100	100
2-h ischemia	100	47 ± 5	46 ± 1
18-h ischemia	51 ± 1	53 ± 6	54 ± 4

Values are given as mean ± SEM.

Also in the 18 h-I group it was not possible to detect IP using LDF. However, after 30 and 60 min there was a slight and after 120 min of reperfusion there was a significant reduction of flux values to 160 ± 13 AU when compared with the control and the 2 h-I group (Fig. 3a). The analysis of RBC velocity by LDF showed a significant reduction at 30 and 120 min of reperfusion. Oscillatory fluctuations in the RBC velocity signal were not detected.

Histology

Short-term preservation of 2 h followed by transplantation (Fig. 5b) did not enhance cell infiltration (6.2 ± 0.8 mm^{-2}, Fig. 4a) or swelling (11.2 ± 2.0%, Fig. 4b) when compared with controls (Fig. 5a), significantly. In the 18 h-I group (Fig. 5c) leukocyte tissue infiltration (51.8 ± 24.1 mm^{-2}, Fig. 4a) was significantly higher when compared with the 2 h-I group and controls (4.8 ± 4.0 mm^{-2}). Interstitial edema formation in the 18 h-I group (17.8 ± 2.2%) was significantly higher when compared with sham-operated controls (4.3 ± 1.3%, Fig. 4b).

Discussion

With the present study we focused on IR-related capillary perfusion alterations in the early reperfusion period of the transplanted pancreas. As the microcirculation in the exocrine tissue of the 2 h-I group exhibited completely preserved FCD, evidence for a so-called primary no-reflow phenomenon [17] was absent in this group. The initial reperfusion period in this group was even characterized by temporary hyperemia and continuous capillary blood flow. As no-reflow is initiated by ischemia and the degree of the microcirculatory alterations correlates with the duration of the ischemic interval [17], two factors apparently contributed to the findings in this group: cold ischemia at 4 °C decisively limited cell damage and prolonged the ischemic tolerance. Furthermore, UW solution effectively suppressed endothelial cell swelling during the relatively short preservation time of 2 h. Hypothermic preservation of 18 h led to a significant

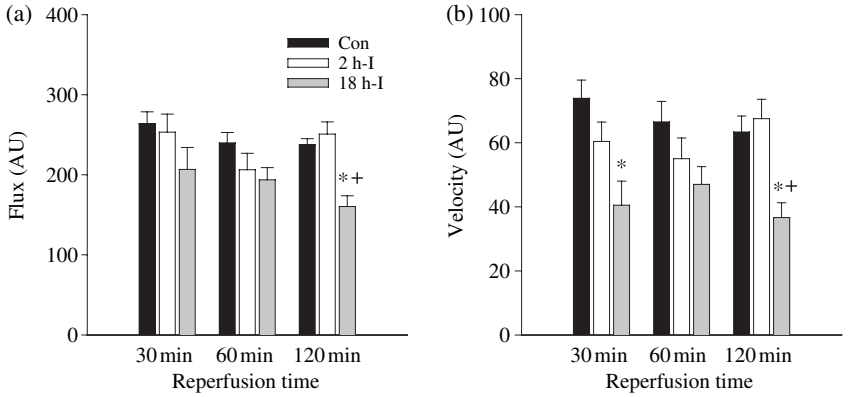

Figure 3 (a) Flux values (AU) and (b) erythrocyte velocity (AU) in exocrine tissue of pancreas grafts after 2-h (white bars) or 18-h (grey bars) hypothermic ischemia as measured by laser Doppler flowmetry. Data shown are arbitrary units and represent mean ± SEM. *$P < 0.05$ versus controls; +$P < 0.05$ versus 2-h ischemia group.

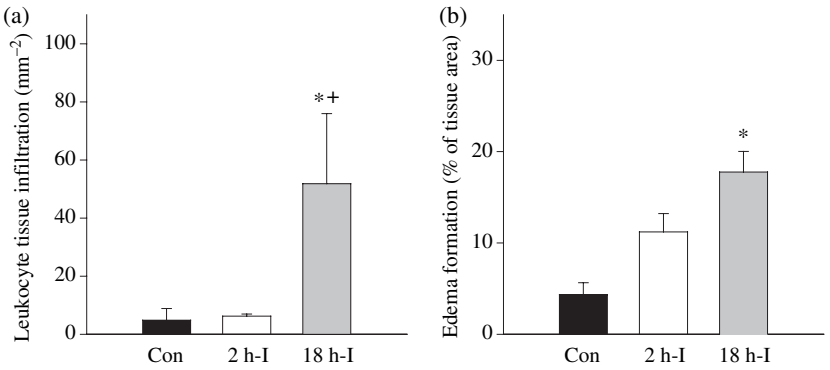

Figure 4 (a) Leukocyte tissue infiltration (mm^{-2}) and (b) edema formation (percentage of total tissue area per high power field) in exocrine tissue of controls (black bars), pancreas grafts after 2-h (white bars) and 18-h hypothermic (grey bars) ischemia as measured by histomorphometric analysis. Data shown represent mean ± SEM. *$P < 0.05$ versus control; +$P < 0.05$ versus 2-h ischemia group.

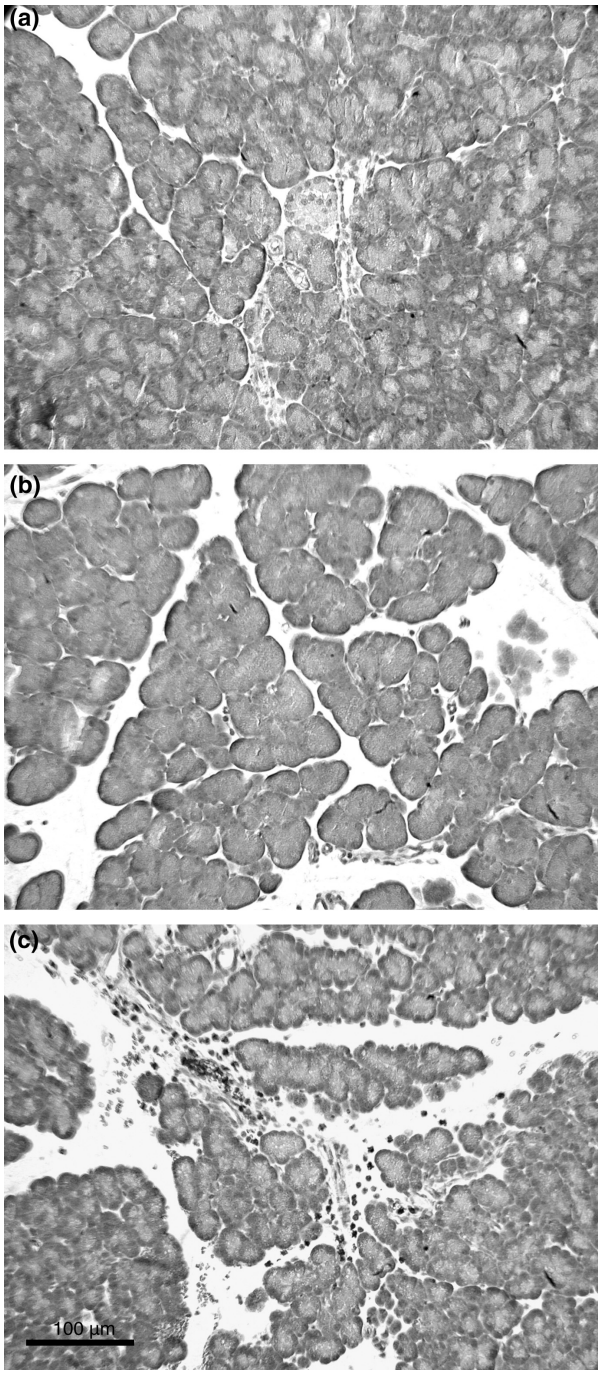

Figure 5 Chloroacetate esterase stained histological sections of a (a) pancreas in the control group, (b) pancreas graft in the 2 h-I group and (c) pancreas graft in the 18 h-I group. Significantly enhanced tissue edema and leukocyte infiltration in the pancreas graft following 18-h hypothermic preservation and 2 h of reperfusion. Bar represents 100 µm.

deterioration of the FCD by 25–30%. As this deficit was present already after 30 min of reperfusion, it can be attributed at least in part to the ischemic tissue damage with primary capillary no-reflow. Although in the ensuing reperfusion period a further significant decrease of the FCD was noted, nutritive perfusion did not fail completely. Apparently, nutritive perfusion in both groups was preserved at excellent or sufficient levels. The extensive prolongation of the preservation period to 18 h led to a severe reperfusion injury; however, protective mechanisms within the pancreas graft obviously counteracted extreme fluid shifts, cell swelling and thus complete perfusion failure. In both transplant groups we observed the induction of IP, an alternating complete cessation and resumption of capillary blood flow: the microcirculation in the 2 h-I group was characterized by the onset of IP in 45% of the ROIs after 60 min of reperfusion. Until the end of the reperfusion period at 120 min IP demonstrated highly constant frequency characteristics and finally extended to almost all observed areas (87%) of the exocrine tissue. Following 18-h long-term preservation an initial period of hyperemia was absent. IP in this group did not exceed >50% of the acinar tissue area. Frequency of IP was shifted to a slightly lower and broader range and within one cycle of 'stop and go' there was a trend towards longer periods of flow when compared with the 2 h-I group.

In contrast, IP was not found in the pancreatic microcirculation of sham-operated controls. This is in line with a previous study, where IP was absent within the normal microcirculation of rat acinar tissue, but was provoked under a low-flow state during hemorrhagic hypotension [8]. The increasing incidence of IP in the course of the reperfusion period – while FCD remained almost unchanged at the same time – emphasizes its role as a regulator of microvascular perfusion in the transplanted pancreas. IP could serve as a compensatory mechanism opposing imminent microcirculatory failure caused by either lowered perfusion pressure or increased hydraulic resistance. Via alternating perfusion of different acini a higher perfusion pressure to single-tissue areas is achieved. Thus, IP provides a spatially adequate tissue blood supply ensuring intermittent oxygenation rather than permanent anoxia [18]. Furthermore, IP could counteract IR-induced pathological fluid shifts. Reversal of the pressure gradient during capillary no-flow periods enables fluid reabsorbtion from interstitial and intracellular compartments [19], resulting both in endogenous hemodilution with improvement of the viscoelastic properties of blood and in reduced tissue pressure and edema formation. In the 18 h-I group this mechanism was operative only in 50% of the ROIs maximally. Consequently, morphometric analysis showed significant edema formation in the 18 h-I group. Furthermore, a high incidence of IP might also have a positive effect on the inflammatory reactions within the graft, as indicated by a

lower leukocyte tissue infiltration in the 2 h-I group when compared with the 18 h-I group.

The lower incidence of IP in the 18 h-I group might indicate a direct damage to the mechanism itself, what is supported by the fact that the frequency range was found to be broader and altered towards longer stop-and-go periods when compared with the highly constant frequency characteristics in the 2 h-I group.

Arteriolar vasomotion, the cyclic constriction and relaxation of small afferent blood vessels, is thought to be the motor of IP in the rat pancreas, because the frequency characteristics were found being almost identical in the two phenomena [8]. Similar frequency spectra in the present study imply that arteriolar vasomotion is the origin of IP also in the transplanted pancreas. Arteriolar vasomotion *per se* additionally contributes to improved tissue perfusion: vascular resistance within arterioles demonstrating vasomotion is reduced when compared with vessels with identical average, but static diameters [20].

About the mechanisms underlying vasomotion and IP only little is known. The incidence of IP during hemorrhagic shock suggested that the trigger was a drastically lowered perfusion pressure: central, sympathetic vasoconstriction, because of low blood pressure, opposing local vasodilation, because of accumulation of acidic metabolites, might have resulted in an oscillatory response eliciting IP [8,21]. Indeed, modulation of vasomotion by sympathetic nerve stimulation could be demonstrated [22]. However, the pancreas grafts were anastomosed to renal vessels of hemodynamic stable animals presenting with a constant arterial blood pressure of 85–100 mmHg throughout the whole observation period in all groups. As perfusion pressure was obviously not lowered and the pancreas grafts were cut off from a central sympathetic supply, another trigger mechanism localized in the graft itself has to be taken into account. Feedback mechanisms in the vasculature between endothelial cells and smooth muscle cells, for example, reacting towards local tissue stimuli like changes of membrane potential, pH, pO_2, Ca^{2+} [23], endothelium-derived factors like nitric oxide [24] or the production of oxygen radicals [25], seem to be involved. Indeed, coupling between endothelial cells, serving as tissue sensors, and smooth muscle cells, serving as effectors, has been reported to be crucial for the induction of vasomotion [26]. Within smooth muscle cells synchronous calcium oscillations via voltage-gated calcium channels were found to regulate vasomotion activity [27,28]. Especially smooth muscle cells at arteriolar bifurcations were proposed to be the pacemakers of vasomotion [29,30].

The effectiveness of UW solution in the preservation of IP seems to be rooted in an excellent endothelial cell protection, as the endothelium is the primary target site of the IR-injury on one hand and plays a key role for the induction of vasomotion on the other. This is in line with a recent study, where UW solution was found to be superior in protecting endothelial cells from ischemic injury, when compared with HTK and Celsior solution [31]: related to the adenosine content of UW solution, ATP levels in endothelial cells remained significantly higher when preserved in this solution than in HTK or Celsior. Furthermore, the potassium concentration of 125 mmol/l in UW solution could contribute to reduced endothelial swelling and better maintenance of the membrane potentials upon IR.

Laser Doppler flowmetry only partially confirmed the data of IVM analysis. Therefore, the sensitivity of this method for the analysis of nutritive perfusion in pancreas grafts seems inadequate when compared with IVM. Contrary to skin [32] or skeletal muscle [7] vasomotion, LDF was not able to detect the onset of IP in the transplanted pancreas either by RBC flux or velocity variations. This might be due to the optical characteristics and high capillary density in the pancreas. Within the 1 mm^3 sample volume of the laser beam, pancreatic acini revealing asynchronous IP might be present, thereby erasing an overall oscillatory signal. Consequently, LDF can be used to grossly compare blood flow in pancreas grafts [33], but it is inferior to IVM for analysis of vasomotion and IP.

Based on our findings we conclude that IP is an important regulator in the microcirculation of the transplanted pancreas aimed at improving tissue blood supply and oxygenation. As its underlying mechanisms can be damaged during hypothermic ischemia, optimal organ procurement strategies should consider the preservation of IP as an important factor. UW solution seems to be suitable to maintain the ability for IP via endothelial cell protection even after long-term preservation.

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