

Tetrahydrobiopterin Attenuates Microvascular Reperfusion Injury Following Murine Pancreas Transplantation

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In this study we investigated the effect of tetrahydrobiopterin (BH4), an essential cofactor for nitric oxide synthases, on ischemia-reperfusion injury (IRI) following murine pancreas transplantation. Pancreatic grafts were exposed to prolonged cold ischemia times (CIT) and different treatment regimens: normal saline (S), S + 16 h CIT, BH4 50 mg/kg + 16 h CIT. Nontransplanted animals served as controls. Graft microcirculation was analyzed by means of functional capillary density (FCD) and capillary diameters (CD) after 2 h reperfusion using intravital microscopy. Quantification of inflammatory responses (mononuclear infiltration) and endothelial disintegration (edema formation) was done by histology (hematoxylin and eosin), and peroxynitrite formation assessed by nitrotyrosine immunostaining. FCD was significantly reduced after prolonged CIT, paralleled by increased peroxynitrite formation as compared with controls (all $p < 0.05$). Microcirculatory changes correlated significantly with intragraft peroxynitrite generation (Spearman: $r = -0.56$; $p < 0.01$). Pancreatic grafts treated with BH4 displayed markedly higher FCD values ($p < 0.01$) and abrogated nitrotyrosine staining ($p = 0.03$). CD were not significantly different in any group. Histology showed increased inflammation, interstitial edema, hemorrhage, acinar vacuolization and focal areas of necrosis after 16 h CIT, which was diminished by BH4 administration ($p < 0.01$). BH4 treatment significantly reduces post-ischemic deterioration of microcirculation as well as histologic damage and might be a promising novel strategy in attenuating IRI following pancreas transplantation.

Key words: Graft pancreatitis, ischemia-reperfusion injury, microvascular injury, nitric oxide synthase, pancreas transplantation

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Introduction

Simultaneous kidney-pancreas transplantation is currently the treatment of choice in patients with diabetes mellitus and end-stage renal disease to achieve long-term normoglycemia and insulin independence (1). Better surgical techniques, novel immunosuppressive regimens and more effective prophylaxis of infections resulted in markedly improved overall results (2).

Nevertheless, ischemia-reperfusion injury (IRI) with consecutive graft pancreatitis is still one of the most threatening complications following pancreas transplantation (3). Various pathophysiological events including microcirculatory disorders, endothelial cell activation, expression of proinflammatory cytokines and adhesion molecules as well as loss of endothelial integrity have been attributed to IRI (4).

Nitric oxide (NO) is a free radical produced by a family of enzymes, the nitric oxide synthases (NOS) (5). NO has been shown to be an important mediator of various biological processes such as vascular homeostasis, neurotransmission and inflammation (6–8). Under physiological conditions NO maintains coronary vasodilatory tone, inhibits platelet aggregation and smooth muscle cell proliferation (9,10). Moreover, it also regulates neutrophil recruitment by inhibiting the expression of adhesion molecules and has negative inotropic and chronotropic effects on cardiomyocytes. Recently, exogenous NO was shown to reduce IRI after solid organ transplantation (11).

However, an excess production of NO by the inducible NOS isoform (iNOS, NOS II) has been demonstrated in many inflammatory processes as well as in tissue reperfusion injury, leading to expression of adhesion molecules,

neutrophil recruitment, increased production of inflammatory cytokines, as well as increased peroxynitrite and superoxide formation (12,13). The precise role of NO generated by iNOS during ischemia and reperfusion is not completely understood and still under debate.

(6R)-5,6,7,8-Tetrahydro-L-biopterin (BH4) is an essential cofactor for the catalytic activity of all NOS isoforms (14). In addition, BH4 has profound effects on the structure of NOS, including the ability to shift its heme iron to a high spin state, increase arginine binding, and stabilize the active dimeric form of the enzyme (15–17). There is also evidence that NOS-bound BH4 may act as a redox-active cofactor by scavenging NOS-derived free radicals (18).

Oxidative stress such as IRI has been demonstrated to deplete intracellular BH4 stores by altering pterin metabolism activity (19,20).

Given the insufficient availability of BH4, the NOS enzyme becomes uncoupled after which the heme group of the enzyme can directly reduce oxygen and release superoxide instead of NO and thereby substantially contributes to oxidative injury following ischemia and reperfusion. Such oxidative stress is thought to play a major role in endothelial activation in transplanted organs (21).

The aim of the present study was to determine whether BH4 would exert beneficial effects on IRI following experimental pancreas transplantation in terms of improved microvascular perfusion, extravasation of inflammatory cells or the generation of oxygen free radicals.

Material and Methods

Animals

Ten- to 12-week-old male C57BL6 (H2^b) mice obtained from Harlan-Winkelmann Co., (Borchen, Germany) were used as size-matched donor and recipient pairs. Animals were housed under standard conditions at the animal center of Innsbruck Medical University and given rat chow and water *ad libitum* before and after transplantation.

All animals received humane care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985), and all experiments were approved by the Austrian Ministry of Education, Science and Culture.

Pancreas transplantation

Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (40–45 mg/kg body weight) and atropine sulfate (0.14 mg/100 g body weight). All surgical procedures were carried out by a single surgeon under clean but not sterile conditions, using an operating microscope with 7–70 × magnification (SZ-STU2, (OLYMPUS Inc., Tokyo, Japan). Cervical heterotopic pancreas transplantation was performed with a modified no-touch technique. Briefly: *Recipient operation*: The right external jugular vein (EJV) and the common carotid artery were dissected free. By using a polyethylene cuff (OD 0.63 mm) it became possible to evert the artery

over the cuff body and finally fix the vessel with 8-0 silk ligatures. Similarly, the EJV was everted over a 0.94 mm cuff. *Donor operation*: After a complete midline incision the pancreas was isolated using a no-touch technique on a segment of the aorta, including the celiac axis and the superior mesenteric artery. Venous outflow was provided by the portal vein. *Implantation*: The graft was placed in the right cervical region and vascular anastomoses completed by pulling the PV over the EJV-cuff and the donor aortic segment over the carotid cuff and held in place with a 8-0 silk ligature. After releasing venous and arterial clamps, all grafts immediately returned to their normal pink color, with the arterial stump pulsating.

Experimental design

Pancreatic grafts were subjected to various cold ischemia times (CIT) and treatment regimens. Warm ischemia time (WIT) was strictly standardized at 45 min in all animals in all groups (n = 5 per group). Group I: 0 h CIT + 45 min WIT + normal saline (S); Group II: 16 h CIT + 45 min WIT + S; Group III: 16 h CIT + 45 min WIT + BH4. Nontransplanted animals served as controls in Group IV. BH4 was obtained from Schircks Laboratories, Jona, Switzerland, and 50 mg/kg body weight was intraperitoneally injected prior to transplantation and immediately after reperfusion in recipient animals in Group III.

Intravital fluorescence microscopy

Intravital fluorescence microscopy (IVM) was used to analyze graft microcirculation by means of functional capillary density (FCD) and capillary diameters (CD) after 2 h reperfusion. IVM was performed using an inverted IX-70 microscope (Olympus, Nagano, Japan) with a 100 W mercury vapor lamp HB-10103AF-Hg. Filterblocks U-MWIB2 for FITC (excitation wavelength 460–490 nm, emission wavelength 510 nm, Olympus) were used for epi-illumination. In order to enhance the contrast of the microvessels 0.3 mL of a 0.4% fluorescein-isothiocyanate (FITC)-labeled dextran (MW 150 000; 50 µg/kg body weight; SIGMA, Deisenhofen, Germany) was injected via the penile vein. Analysis was performed with a charge-coupled camera device (Kappa opto-electronics Inc. Monrovia, CA, USA), and the images were acquired using KAPPA Image Base software. Quantitative image analysis was performed with PicEd Cora (JOMESA, Munich, Germany) software.

Histology

For histological examination, grafts were fixed in 10% formaldehyde for 24 h, embedded in paraffin, and stained with hematoxylin and eosin (H&E). According to Schmidt's method, a pathologist blinded to the treatment protocol scored the tissues for inflammatory infiltration, edema formation, parenchymal necrosis and hemorrhage (22).

Immunohistochemistry

Graft sections (4 µm) were cut from paraffin blocks, mounted on slips and the paraffin removed by heating in citrate buffer, pH 6.0. Endogenous peroxidase was blocked with hydrogen peroxide 0.3%. Immunohistochemistry was then performed in a diaminobenzidine tetrahydrochloride (DAKO) autostainer (DAKO, Copenhagen, Denmark), using an antinitrotyrosine rat polyclonal antibody from Upstate Biotechnology (Lake Placid, NY, USA) at 1:100 dilution. For staining, secondary antibody peroxidase-labeled polymer and 3,3' DAKO were used. Haemalaun was used for counterstaining. For quantification, the product of proportion of positive cells in quartiles (0, 1, 2, 3, 4), and the staining intensity (0 no staining; 1 weak; 2 moderate; 3 strong) was calculated, yielding a total immunostaining score ranging from 0 to 12.

Analysis of plasma nitrite plus nitrate

Plasma nitrite plus nitrate was determined with a method modified from Green et al. (23). Protein was removed by ultrafiltration (10 kDa cut-off, Microcon 10, Millipore, Vienna, Austria) and then analyzed by HPLC

(20- μ L injection volume) including cleanup on a reversed-phase C-18 column (125 mm long, 4 mm i.d., 5- μ m particle size, Lichrocart, Merck, Darmstadt, Germany, eluted with 2% (w/v) ammonium acetate, pH 7.0, flow rate 0.7 mL/min). This was followed by reduction of nitrate to nitrite on a cadmium (Merck) reactor column, post-column mixing with the Griess reagent (flow rate 0.7 mL/min), heating in a 10 m capillary at 60°C and detection of the azo-dye at 546 nm in a UV/Vis detector (UV 200, Varian, Vösendorf, Austria).

BH4 tissue levels

For determination of BH4 concentrations, tissue was homogenized on ice with a microblender in distilled water containing 5 mM dithioerythrol, centrifuged at 12 000 g at 4°C for 10 min and then subjected to oxidation in acid or base, by a method modified from Fukushima et al. (24). To 100 μ L supernatant, 20 μ L containing 0.5 M HCl and 0.05 M iodine were added for acidic or 20 μ L 0.5 M NaOH plus 0.05 M iodine for basic oxidation. After incubation for 1 h in the dark at room temperature, 20 μ L HCl was added to the basic oxidation only, and all mixtures received 20 μ L 0.1 M ascorbic acid for the reduction of excess iodine. Samples were then centrifuged for 10 min at 12 000 g and 4°C. Biopterin concentrations were determined by HPLC using 10- μ L injection volume, a Nucleosil 10 SA column (250 mm long, 4 mm i.d., Macherey Nagl, Düren, Germany), eluted with 1.5 mL/min 50 mM potassium phosphate buffer, pH 3.0 and fluorescence detection (excitation 350 nm, emission 440 nm). BH4 concentrations were calculated as difference of results from oxidation in acid and base, respectively.

Serum amylase levels

Serum amylase levels were determined by standard laboratory methods using a Hitachi automatic analyzer (Boehringer Mannheim, Mannheim, Germany).

Statistics

Results are expressed as mean \pm SEM. Statistical analysis was performed with SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). When two groups were compared, a 2-tailed Student's *t*-test was utilized. The Spearman rank coefficient correlation test was used to examine the relationship between

FCD and nitrotyrosine immunostaining. A *p* value of <0.05 was considered to be of statistical significance.

Results

BH4 tissue levels

Total biopterin and BH4 tissue levels in pancreas grafts were assessed by HPLC. Values are expressed as mean \pm SEM (*n* = 5 animals per group) in pmol/ μ g protein. No significant differences were seen in total biopterin levels between groups of nonischemic controls (Group IV: 13.6 \pm 3.9), and those with 45 min WIT (Group I: 13.3 \pm 6.6) and 16 h CIT (Group II: 19.0 \pm 5.9). Treatment with BH4 was associated with a massive increase in intragraft biopterin concentrations (Group III: 405.5 \pm 203.6) (Figure 1A). The high variability in the BH4 treated group was caused by animal-to-animal variation in response to the BH4 treatment rather than by variability in the measurement method, which showed a coefficient of variation of 2.6 \pm 0.7% (mean \pm SD of triplicate variation of pancreas samples from seven BH4-treated animals). Prolonged CIT, however, significantly decreased BH4 concentrations expressed as the percentage of total biopterin to 58.2 \pm 16.7% as compared to nonischemic controls (94.2 \pm 2.3%). This was restored to 80.1 \pm 6.6% by systemic supplementation of BH4 (Figure 1B).

Analysis of graft microcirculation: After 2 h reperfusion, pancreas grafts were retrieved for IVM. Prolonged CIT (Figure 2C) caused remarkable perfusion deficits with leakage of the capillary mesh, as compared to the homogeneous perfusion patterns of baseline controls and after

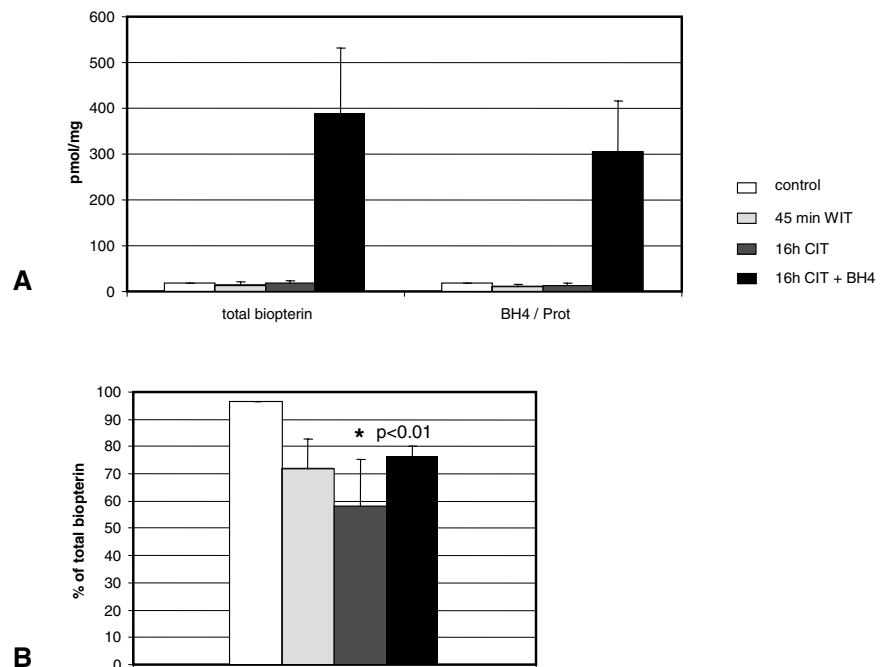


Figure 1: BH4 tissue levels. (A) Total biopterin tissue levels in pancreas grafts after 2 h reperfusion. Data are expressed as pmol biopterin per mg protein. (B) BH4 concentrations as percentage of total biopterin. **p* < 0.01 as compared with controls and after BH4 supplementation. Data are means \pm SEM.

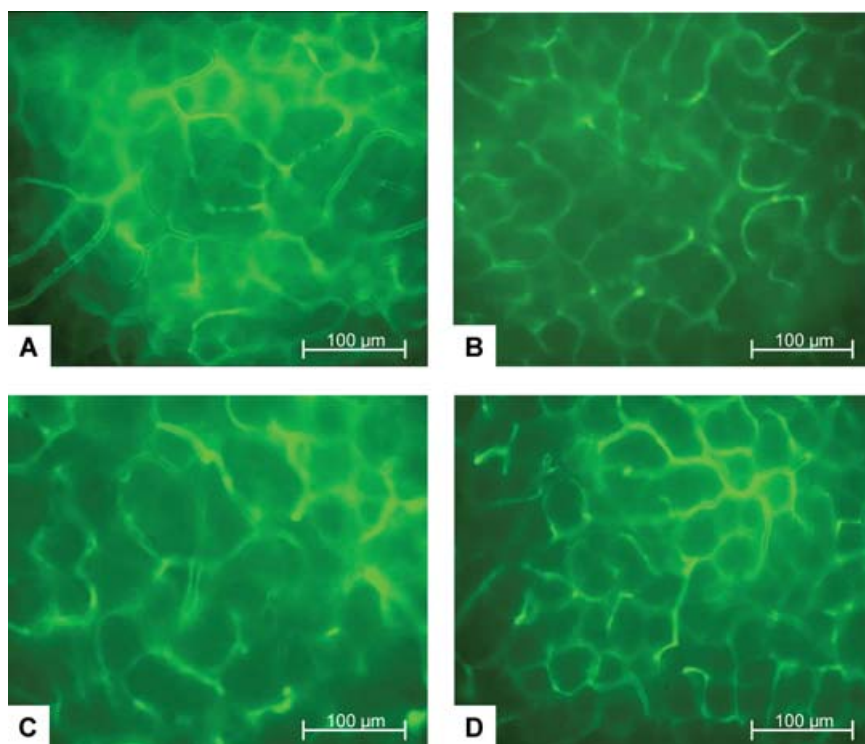


Figure 2: Intravital fluorescence microscopic images. (A) Physiologic pancreatic microcirculation in non-transplanted controls without IRI lacking any signs of microvascular injury. (B) Only minimal microvascular impairment was evident following 45 min WIT. By contrast (C) displays severe perfusion deficits in grafts after prolonged CIT. (D) shows reversal of microcirculatory changes due to BH4 supplementation. Original magnification: $\times 350$.

45 min WIT alone (Figure 2A,B). Pancreas grafts treated with BH4 (Figure 2D), by contrast, displayed marked improvements and almost reversal of microcirculatory changes due to prolonged cold ischemia.

To quantify microvascular injury we determined FCD, which is defined as the length of all blood cell-perfused nutritive capillaries per observation area. Mean FCD in baseline controls (Group IV) was $355 \pm 6.7 \text{ cm}^{-1}$ which was only slightly decreased to $301 \pm 54.5 \text{ cm}^{-1}$ following 45 min WIT (Group I). However, 16 h of CIT (Group II) caused a significant and persistent reduction in graft microcirculation reflected in an FCD of $228 \pm 44.8 \text{ cm}^{-1}$. In parallel, we observed overall heterogeneity of microperfusion with an increase in intercapillary distance and CD, as well as scattered microvascular thrombosis and hemorrhage in grafts subjected to prolonged cold ischemia (data not shown).

BH4 treatment (Group III) significantly increased microcirculatory parameters resulting in an improvement in FCD of $303 \pm 21.8 \text{ cm}^{-1}$ which was comparable to baseline controls and grafts without cold ischemia (Figure 3). Furthermore, no signs of microvascular thrombosis or hemorrhage were recognized in BH4-treated grafts. Macroscopically, all grafts subjected to prolonged cold ischemia revealed severe morphological changes such as marked edema and areas of subcapsular hemorrhage, whereas after BH4 supplementation pancreatic grafts reperfused immediately and were homogeneously pink in color (data not shown).

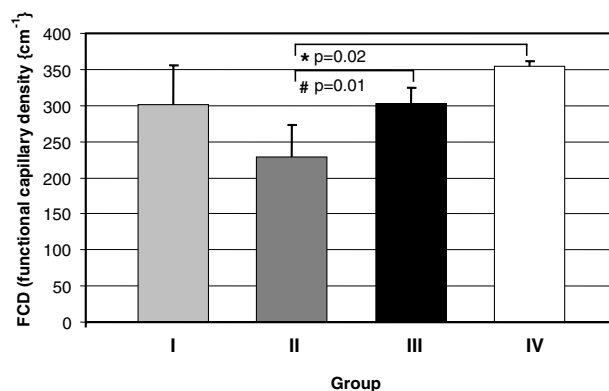
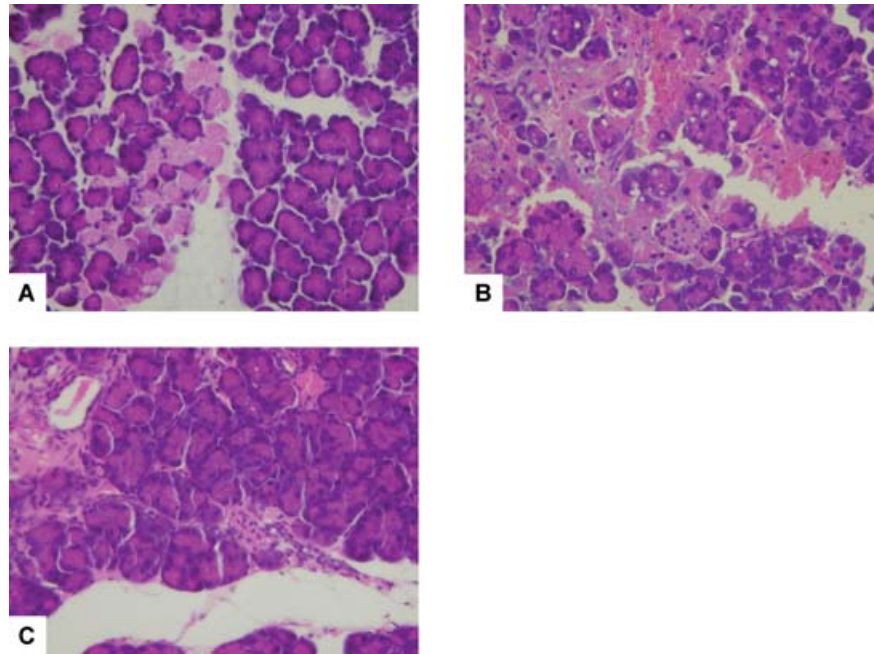


Figure 3: Quantification of microvascular injury. Functional capillary density of murine exocrine tissue after whole pancreas transplantation assessed by IVM according to different experimental groups. Group I, 45 min WIT. Group II, 16 h CIT. Group III, 16 h CIT + BH4 supplementation. Group IV, nontransplanted animals with only exteriorization of the pancreas. Data presented as means \pm SEM. (* $p = 0.02$ vs. Group IV; # $p = 0.01$ vs. Group III). Group I vs. Group II, $p = 0.05$; Group III vs. Group IV, $p = \text{n.s.}$; Group I vs. Group III, $p = \text{n.s.}$; Group I vs. Group IV, $p = \text{n.s.}$

Graft histology: Histological findings determined by H&E staining were classified using Schmidt's score, which reflects quantification of interstitial edema, acinar necrosis, hemorrhage, necrosis and graft inflammation.

Figure 4: H&E histology 2 h after reperfusion. (A) Nontransplanted controls. (B) Increased interstitial edema, acinar vacuolization and necrosis as well as leukocyte infiltration after prolongation of graft storage to 16 h. (C) displays significant amelioration of CIT-induced histological damage in BH4-treated pancreatic grafts. (D) Semi-quantitative histological score of graft pancreatitis including edema, acinar cell necrosis, hemorrhage and inflammatory infiltration. In animals with BH4 supplementation, all these changes were significantly attenuated (*all $p < 0.01$ vs. Group II; magnification $\times 200$).



As compared to 45 min WIT (Group I) (Figure 4A), prolonged cold ischemia (Group II) resulted in morphologic changes including aggravated inflammation (neutrophil cell infiltration), interstitial edema, hemorrhage, acinar vacuolization and focal areas of necrosis (Figure 4B). Histology of pancreas grafts treated with BH4 (Figure 4C) revealed significantly diminished changes with only slightly interstitial edema and vacuolization in acinar cells. BH4 thereby also significantly reduced the quantitative scores for pancreatic graft tissue damage (Figure 4D).

Peroxynitrite (ONOO⁻) formation: The nitrosilation of tyrosine is paradigmatic for the reaction of ONOO⁻ with aromatic amino acids and is therefore an indirect marker for this strong oxidative agent. Immunostaining for nitrotyrosine was thus performed to indirectly estimate peroxynitrite generation (Figure 5A–C). As compared to nontransplanted controls (Figure 5A), nitrotyrosine staining was strongly increased after prolonged cold ischemia (Figure 5B). This was prevented by BH4 treatment (Figure 5C).

For quantification purposes the product of the proportion of positive cells and the staining intensity was calculated yielding a total immunostaining score ranging from 0 to 12 (see Material and Methods).

Sixteen hours CIT (Group II) revealed an immunostaining score of 7.0 ± 1.73 , which was significantly higher than that after 45 min WIT (Group I, 4.67 ± 1.15) or in nonischemic control pancreata (Group IV, 3.33 ± 1.15). BH4 treatment significantly attenuated the increased pro-

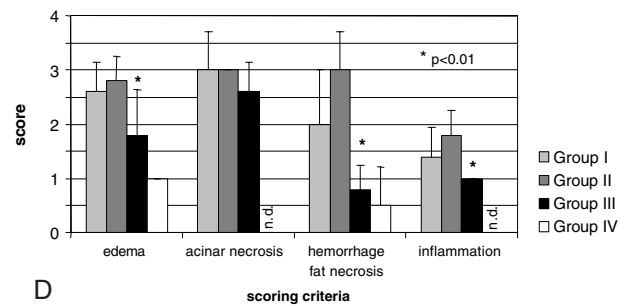


Figure 4: Continued.

duction of ONOO⁻ due to prolonged CIT and overall scores were comparable with those of baseline controls (Figure 5D). In addition, intra-graft peroxynitrite generation correlated significantly with the impairment of pancreatic microperfusion and FCD scores (Spearman: $r = -0.56$; $p < 0.01$; data from Figures 3 and 5D).

Serum amylase levels

Serum amylase levels are widely accepted as an indicator of acinar cell necrosis and subsequent pancreatic tissue injury (25). Amylase levels were significantly increased in all experimental groups following 2 h of reperfusion (Group I: 9429 ± 1727 U/L; Group II: 5300 ± 1368 U/L; Group III: 6036 ± 1153 U/L) as compared to nonischemic controls in Group IV: 2304 ± 360 U/L; all $p < 0.001$. However, despite profound effects on microcirculatory parameters and peroxynitrite, BH4 therapy did not show beneficial effects

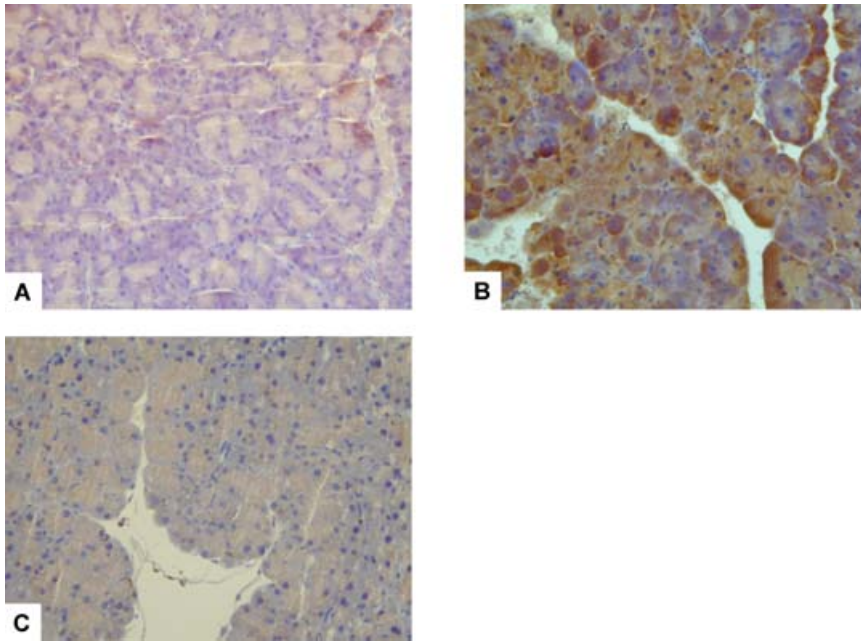


Figure 5: Immunostaining for nitrotyrosine. (A) Nontransplanted controls, (B) Prolonged cold ischemia time resulted in prominent intragraft nitrotyrosine staining with strong cytoplasmic reactivity. (C) This staining was abolished by treatment with BH4. Magnification $\times 200$. (D) Quantitative immunostaining score as the product of the proportion of positive cells and the staining intensity (see Material and Methods). * $p = 0.03$ vs. Group II.

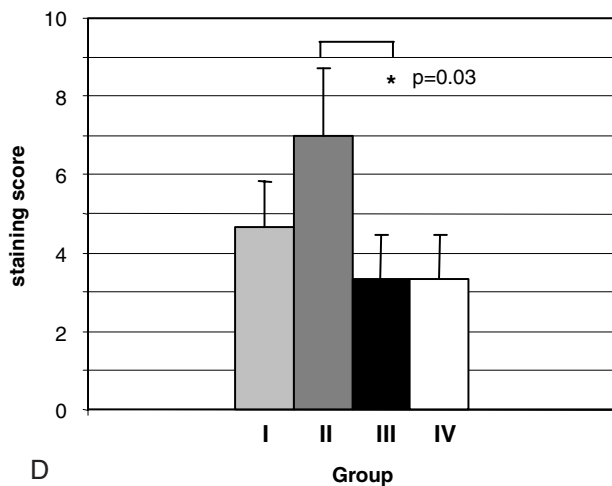


Figure 5: Continued.

in terms of inhibition of pancreatic exocrine secretion as reflected by unchanged serum amylase in this model.

Discussion

In the present study we were able to demonstrate that BH4 supplementation attenuates IRI caused by 16 h cold ischemia followed by 2 h reperfusion in a murine pancreatic transplant model. Moreover, BH4 also restores micro-circulatory defects due to prolonged cold ischemia, diminishes histological changes like interstitial edema, acinar vacuolization and focal necrosis and attenuates peroxynitrite formation. Consequently, our results clearly indicate

that BH4 exerts protective effects against post-ischemic injury following experimental pancreas transplantation.

It has previously been shown that availability of BH4 is essential for the catalytic activity of NOS (26). Given inadequate concentrations of BH4, the NOS enzyme becomes uncoupled after which superoxide anions and peroxynitrite are released instead of NO and thereby substantially contribute to oxidative injury following ischemia reperfusion (27). Inflammation as well as reperfusion injury may limit BH4 availability, either by altering pterin metabolism or by increasing oxyradical stress (20). In particular, the endothelium is considered to generate free radicals during IRI. Furthermore, oxyradical injury alters the redox state of endothelial cells and thus impairs BH4 availability, since the biosynthesis of BH4 depends on a normal cellular redox state (28). In the present study tissue BH4 concentrations were seen to be significantly decreased following prolonged cold ischemia in murine pancreatic grafts as compared to grafts without cold ischemia; this was sufficiently reversed by exogenous supplementation. BH4 is one of the most powerful naturally occurring reducing agents, and it is therefore reasonable to hypothesize that BH4 might be a molecular target for oxidative stress resulting in BH4-depletion due to excessive oxidation. In line with these results, Cosentino and Lüscher (26) reported that eNOS serves as a source of oxygen free radicals in coronary arteries depleted of BH4, and Hishikawa et al. (29) showed that inhibition of BH4 synthesis increased superoxide anion production. NOS can therefore be regarded as dysfunctional under certain conditions and hence becomes a free oxygen radical (superoxide anion, peroxynitrite) instead of a protective NO-producing enzyme, a shift further triggered by reduced BH4 levels. However, the exact

mechanism underlying the decreased availability of BH4 was not clarified in the current study and needs further investigation. We hypothesize that toxic radicals generated during IRI might either affect the biosynthesis of BH4 or prevent BH4 recycling. Unfortunately, the levels of active and inactive BH4 metabolites cannot be discriminated in tissue. However, the fact that exogenous BH4 significantly attenuated IRI strongly suggests a functional deficiency of BH4 in the graft following ischemia and reperfusion.

A close correlation between the duration of cold ischemia and the severity of graft tissue damage has been reported previously (30). Consequently, we chose a CIT of 16 h to generate a rather severe degree of injury in all pancreas grafts and in order to mimic a most likely clinical situation.

One of the most important features of post-transplant graft pancreatitis is ischemia reperfusion-related disturbances in microvascular perfusion with subsequent endothelial dysfunction, enhanced leukocyte-endothelial interaction and hypoxic tissue damage (31). IVM showed significant microvascular impairment and persistently reduced FCD in the early period after reperfusion due to prolonged cold ischemia and thereby confirmed data from previous studies of rat pancreas transplantation (32,33). However, to the best of our knowledge this is the first study addressing the impact of cold ischemia and subsequent reperfusion, in particular in terms of microcirculatory changes in murine pancreatic grafts. In addition, this is also the first report to reveal a beneficial effect of BH4 supplementation on IRI following pancreas transplantation. Several previous studies have demonstrated that BH4 reduces tissue injury following IRI after kidney, liver, lung and heart transplantation (34–37). In the present study BH4 not only attenuated microvascular perfusion deficits, but also significantly decreased peroxynitrite (ONOO⁻) formation, as reflected by diminished nitrotyrosine staining scores. ONOO⁻ is produced by the rapid interaction of NO with superoxide anions and has been associated with numerous injurious effects on cellular and tissue function including increased oxidative reactions, DNA cleavage, lipid peroxydation and reduction of plasma antioxidants (38,39). Nitration of protein tyrosine residues leads to the formation of 3-nitrotyrosine, which may be considered a marker of ONOO⁻-dependent oxidative damage (40). The generation of oxygen free radicals is increased during reperfusion, depending on the severity and duration of the preceding ischemic period and subsequently might also increase ONOO⁻ formation (41). In line with this suggestion, prolonged cold ischemia in our study revealed a significant increase in nitrotyrosine staining as compared with nonischemic control grafts. Furthermore, our results showing decreased nitrotyrosin immunostaining due to BH4 treatment indicate that BH4 prevents the tyrosine-nitrating properties of peroxynitrite following IRI. Therefore, BH4 may be considered to be protective against intragraft manifestations of oxidative and nitrosative stress in this experimental murine pancreas transplant model. However, despite such protective effects on microcircula-

tory parameters and peroxynitrite formation, BH4 supplementation did not affect levels of pancreatic exocrine enzymes. A possible explanation for unchanged serum amylase levels could be the relatively short observation period of 2 h following reperfusion in this study.

The regulation of NOS gene expression during ischemia and reperfusion is not fully understood. Several studies have indicated that ischemia decreases eNOS expression in endothelial cells of various origin (42,43). On the other hand, however, it has been reported that hypoxia increases eNOS and iNOS expression in various organs and cell types (44,45). In a model of hepatic IRI, iNOS expression was shown to be significantly upregulated following BH4 treatment after 12 h reperfusion (36), whereas Gess et al. reported that hypoxia fails to induce iNOS in rat liver, lung and kidneys (46). Such discrepancies in the expression patterns of NOS among these studies may be due mainly to differences in the experimental set-up or in the duration of ischemia. However, BH4 has been shown to stabilize NOS (eNOS and iNOS) in its active dimeric form (47, 48). Therefore, BH4 may determine the effective amount of NOS isoforms regardless of the total quantity of NOS expression.

In summary, our results suggest that exogenous BH4 supplementation is able to attenuate IRI following pancreas transplantation by significantly decreasing microvascular changes, histologic damage and peroxynitrite formation and suggest a potential therapeutic role for BH4 in the prevention of early graft pancreatitis.

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