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P. Brenner
M. Hinz
H. Huber
M. Schmoeckel
H. Reichenspurner
B. Meiser
C. Hammer
B. Reichart

Influence of ischemic time on hyperacute xenograft rejection of pig hearts in a working heart perfusion model with human blood

P. Brenner (✉) · M. Hinz · H. Huber · M. Schmoeckel · H. Reichenspurner · B. Meiser · B. Reichart
Department of Cardiac Surgery, Klinikum Grosshadern, Ludwig-Maximilians-University of Munich, Marchioninistrasse 15, D-81377 Munich, Germany
e-mail: Paolo.Brenner@hch.med.uni-muenchen.de
Tel.: + 49-89-70954404
Fax: + 49-89-70958897

C. Hammer
Institute for Surgical Research, Klinikum Grosshadern, Ludwig-Maximilians-University of Munich, D-81377 Munich, Germany

Abstract In xenotransplantation long ischemic time of grafts is supposed to have a marked influence on hyperacute rejection (HXR). We investigated the influence of different cold ischemic times on HXR of ex vivo "working pig hearts" perfused with human blood. Xenoreactive natural antibodies (XNAb) as a trigger of HXR were eliminated by Ig-Therasorb immunoadsorption (IA). Explanted Landrace pig hearts of group G1 and group G3 (with additional IA) underwent 4 h of cold ischemia prior to xenoperfusion. Control groups G2 and G4 (with IA) were kept ischemic for only 46.6 ± 15.8 and 51.2 ± 4.2 min, respectively. Ischemic time prolonged the perfusion time in our working heart model (G1: 356 ± 46.1 min; G2: 125 ± 31 min; $P < 0.05$). IA had no additional impact on perfusion time but was effective by itself. The heart weight increased fourfold more in G2 as compared to the other groups. IA without ischemia significantly improved cardiac output in G4 (G3: 198.8 ± 15.4 mL/min; G4: 338.5 ± 16.0 mL/min). Coronary flow in G2 was significantly lower than in G1 (G1: 157.9 ± 9.15 mL/min; G2: 59.4 ± 20.1 mL/min). Histological signs of HXR (light and electron microscopy) could be found in G2 in contrast to the other groups. Parameters of serological damage showed a minimum in G4 and the maximum in G2. In G1 XNAb were nearly equally eliminated immediately after the start of xenoperfusion as in IA groups G4 and G3. Four hours of ischemic time showed beneficial effects in preventing HXR, possibly caused by changes of the endothelial cell surface (for example, glycosylation or loss of α1–3Gal epitopes with a hapten effect).

Key words Ischemic time · Hyperacute xenograft rejection · Xenotransplantation · Ig-Therasorb column · Immunoadsorption

Introduction

A vascularized organ, such as a pig heart or kidney, transplanted into an unaltered, phylogenetically widely disparate recipient, such as a man or non-human primate (discordant xenotransplantation), is invariably rejected within minutes to a few hours [1–3]. This process, known as hyperacute xenograft rejection (HXR), is known to be initiated by the binding of cytotoxic xenoreactive natural antibodies (XNAb) mainly to the carbohydrate structure Galα1–3Galβ1–4GlcNAc-R [4–6] on porcine endothelial cells. The deposition of these antibodies on the graft has the capacity of triggering the activation of the recipient complement cascade [7, 8], which ultimately leads to irremediable destruction of the graft [9, 10] by development of interstitial edema and hemorrhage, the formation of platelet and fibrin thrombi, and by infiltration of polymorphonuclear leukocytes into the graft [11, 12]. The critical role of complement activation has been demonstrated in various models in which comple-

ment depletion or deficiency allowed a prolonged survival of vascularized xenografts [7, 8, 13].

Ischemia–reperfusion injury leads to the activation and deposition of complement on the vascular endothelium [13–15]. In experimental and clinical myocardial ischemia [16–19] it was demonstrated that complement becomes selectively activated in areas of infarction only. Inhibition of complement activation or depletion of complement proteins prior to reperfusion has been shown to reduce tissue injury [20, 21]. In a rat model of myocardial infarction, ischemia and reperfusion injury activated both pathways of the complement system [22]. Thus ischemia and reperfusion injury after prolonged ischemic time (IT) are supposed to have a marked impact on the rejection mechanisms especially with regard to the complement cascade which is activated during HXR after xenotransplantation. Contradictory results have been published in allogenic transplantation regarding the influence of prolonged cold IT on allograft function and rejection. Chronic rejection of rat hearts after allogenic transplantation is accelerated by long IT [23]. Ischemic injury after transplantation of a rat lung did not correlate with onset and intensity of graft rejection [24]. In clinical investigations no correlation was found between IT and the incidence of primary graft failure after pediatric heart transplantation [25]. In addition a persisting sinus node dysfunction did not correlate with IT [26]. No scientific data exist indicating whether ischemia and reperfusion injury after a long IT have a marked influence or a kind of trigger function on HXR. Presuming, that a long IT accelerates antibody-mediated HXR, we selected two additional groups, in which HXR was tried to be prevented. In these groups XNAb as a trigger of HXR were eliminated by immunoadsorption (IA) using the Ig-Therasorb column. In previous experiments IA could effectively prevent HXR [27]. In this study we compared effects of IT on HXR with and without IA. Therefore, we investigated the influence of different cold ITs (two groups with 1 h IT and two groups with 4 h IT) on HXR of ex vivo "working pig hearts" perfused with human blood. The additional effect of IA on antibody-mediated processes in HXR after long IT should be demonstrated. Long IT was defined as 4 h of 4 °C cold preservation time of pig hearts after cardioplegia with Celsior solution because this represents the upper limit of accepted IT in clinical allotransplantation. An irrelevant or short IT was defined as a period of less than 1 h.

Materials and Methods

Immunoadsorption

In the xenogeneic system pig-to-man simulated in our perfusion model, 500 ml human blood from healthy volunteers (representing a xenograft recipient) was treated with IA using the Ig-Therasorb column (Baxter/Therasorb, Unterschleissheim, Germany). Human plasma was passed through a sterile and pyrogen-free glass column (volume 300 ml) containing polyclonal sheep anti-human immunoglobulin antibodies (heavy-chain and light-chain specific) bound to sepharose beads. The antibodies were directed specifically against human immunoglobulins IgG (subclasses 1–4), IgM, and IgA, circulating immune complexes, and fragments of immunoglobulins. In the IA system a hemopump (EP12) with a plasma filter separates human whole blood by plasmapheresis into the plasma and the cellular fraction. A second pump circuit (BM11) ensures a constant plasma flow directed to a pair of Ig-Therasorb columns. After passage through the first adsorption column, the bound immunoglobulins are detached from the sepharose beads by a glycine buffer, pH 2.8, and a PBS buffer solution, pH 7.2. The first absorbing column is regenerated by glycine and PBS solution, while the second column is loaded. Using this special desorption procedure the column becomes re-usable.

Animals

Donor animals were Landrace pigs with a body weight between 13 and 31 kg. Donor pigs were anesthetized with azaperon, ketamine hydrochloride, and xylazine. A tracheotomy was performed and the animals were ventilated mechanically. Anesthesia was maintained with N_2O/O_2 (2/4 l per min), intravenous pancuronium, and fentanyl citrate. After median sternotomy and intravenous injection of heparin (400 IU/kg), cardioplegic arrest was induced with cold Celsior solution and external topical cooling. The heart–lung block was excised, both venae cavae and hili were ligated, and both lungs were removed. Cannulas for the xenoperfusion circuit were inserted into the aortic root and left atrium. A cannula in the pulmonary artery enabled direct measurement of the coronary sinus flow. After connection of the hearts to the working heart apparatus, perfusion was started after different cold ITs according to the protocol.

In total 24 pig hearts were explanted and four series of experiments were performed. In group G1 ($n = 6$), hearts underwent 4 h of ischemia prior to xenoperfusion. In a control group G2 ($n = 6$), IT was only 46.6 ± 15.8 min on average. In group G3 ($n = 6$) with 4 h of IT and in group G4 ($n = 6$) with 51.2 ± 4.2 min of IT, two cycles of IA removed immunoglobulins IgG, IgM, and IgA from the circulating blood prior to reperfusion.

Cardioplegia

To reduce ischemic injury as much as possible we used 200 ml iced (4 °C) low-potassium Celsior solution (Imtix Sangstat, Leimen, Germany) for cardioplegia (contents: 15 mmol/l potassium, 100 mmol/l sodium, 13 mmol/l magnesium, 0.26 mmol/l calcium, 41.5 mmol/l chloride, 30 mmol/l histidine, 80 mmol/l lactobionate, 60 mmol/l mannitol, 20 mmol/l glutamate; osmolarity: 360 mosm/l; pH 7.3 at 20 °C. In this solution histidine serves as a buffer substance. In ex vivo perfusion models with the problem of increasing potassium levels, a low-potasium solution such as Celsior was advantageous.

Ex vivo "working heart" apparatus

In our working heart perfusion model (Fig. 1), earlier decribed by Forty et al. [28] and Suckfüll et al. [29], the blood was transported during xenogeneic reperfusion time (30 min) in the Langendorff mode from the main reservoir by a roller pump (BP 742; Fresenius,

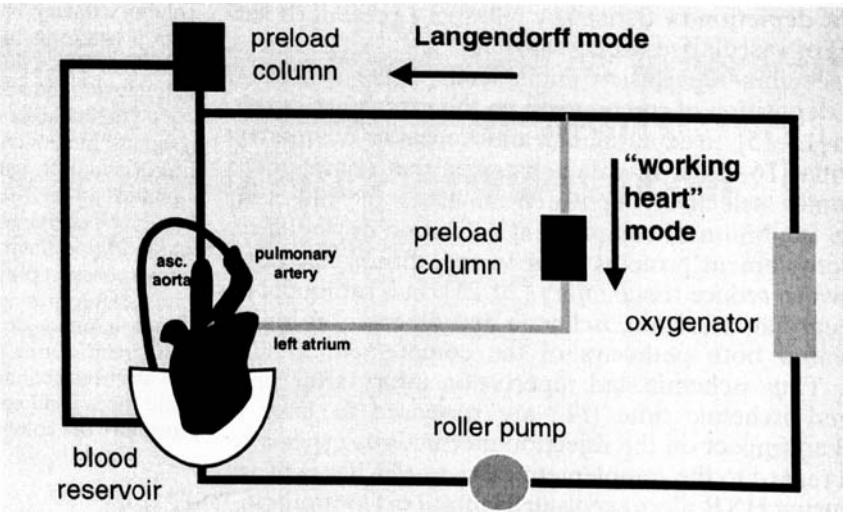

Fig. 1 Working heart model. During reperfusion in the Langendorff mode (30 min), blood is pumped into an afterload reservoir connected to the aortic root for coronary artery perfusion. In the "working heart" mode the left ventricle ejects blood into an afterload column (75 cm). From there blood spills over to a main reservoir. Blood collected in the reservoir is pumped into a temperature-controlled oxygenator and then back to a preload reservoir 15 cm above the left atrium

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Bad Homburg, Germany) through a hollow fiber pediatric oxygenator with integrated heat exchanger (Dideco module 1500; Dideco, Puchheim, Germany) into an afterload reservoir (height 75 cm), which was connected to the ascending aorta. After switching to the working heart mode, blood was collected in the main reservoir and was pumped to a preload reservoir (height 15 cm) connected to the left atrium. The left ventricle was filled at a preload pressure of 15 cm H_2O and ejected blood into the afterload column.

Blood temperature (37 °C) and blood gases were kept within the physiological range controlled online with a pH meter (WTW, Wuppertal, Germany). Glucose (4 mg/h), insulin (15 IU/h), and calcium gluconate (50 mg/h) were substituted continuously. According to the experimental protocol for evaluation of parameters influencing the working heart apparatus, two control experiments (perfusion of the system with pig blood only and autologous perfusion of the pig heart with pig blood) were performed.

Hemodynamic parameters

Working heart perfusion allows a direct measurement of cardiac output (CO) and coronary flow (CF) by collecting the overflow from the arterial reservoir and CF from the coronary sinus effluent via the pulmonary artery. Arterial blood pressure, preload pressure, and heart rate were monitored with a transducer (Gould P 23 ID; Gould, Cardiovascular Products Division, Oxnard, Canada) and visualized on a monitor (Sirecust 308d; Siemens, Erlangen, Germany). From these data the following parameters were calculated. Stroke-work index (SWI) was calculated from [(mean arterial pressure – preload) × CO × 1333)] ÷ (heart rate × heart weight). Coronary resistance (arbitrary units) = mean arterial pressure ÷ coronary sinus effluent, and specific coronary flow (SCF) = CF ÷ heart weight were determined accordingly.

Serology

From the effluent, blood samples were collected within fixed periods of time (initially after 5, 10, 15, 30, 60, 90, 120 min and later hourly) during perfusion to determine immunoglobulins (IgA, IgG, IgM) and complement components (C3, C4). As markers for myocardial damage, creatine kinase (CK and CK-MB), lactate dehydrogenase (LDH), and glutamic-oxalacetic transaminase (GOT) were determined by standard methods. For anti-pig antibody monitoring, porcine plasma (0.5 ml) was serially diluted. Washed pig red blood cells were incubated for 30 min and hemagglutination was ascertained under a light microscope.

Histology

For histological and immunohistochemical investigations, tissue was sampled from several areas of the left and right ventricle and the atria after cardiac arrest. Frozen tissue sections of 4–6 µm were stained using hematoxylin and eosin and examined under a light microscope. For immunohistochemical analysis, cryostat-prepared tissue specimens were stained with FITC-conjugated goat antibodies specific for C3, C4, and C5b-9. Tissue deposits of IgA, IgG, and IgM were stained according to the avidin-biotin method. Monoclonal antibodies were obtained from Dako (Hamburg, Germany) and Immunotech Diagnostics (Marseille, France).

For transmission electron microscopy, tissue sections were embedded in Tissue Tek (Miles, USA), snap-frozen in liquid nitrogen, and stored at –70 °C until use. Other tissue samples were fixed in glutaraldehyde 6.25% and stored until further saccharose (0.2 mol/l) processing. Sections (0.5 µm) prepared with Epon resin were first stained with toluidine–methylene blue in order to gain an overview by light microscopy. Ultra-thin tissue sections of interest (100 nm) were laid on copper grids and stained with uranyl acetate and lead. The examination with transmission electron microscopy (Philips 300) was performed at two magnifications (× 10.000 and × 16.000).

Statistical analysis

To establish statistically the significance of 4 h IT and additional IA, parameters from the control group G2 (without 4 h IT and without IA; $n = 6$) were compared with groups G1 (with 4 h IT and without IA; $n = 6$), G4 (without 4 h IT, but with IA; $n = 6$), and G3 (IT and IA; $n = 6$). The results are given as mean ± SEM. The Wilcoxon test for paired samples was used to compare data

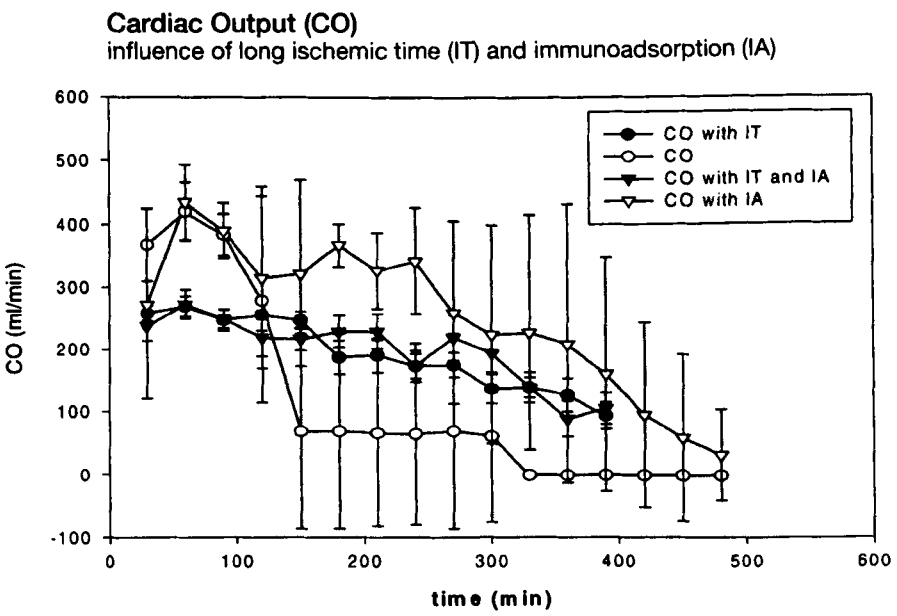


Fig. 2 The best cardiac output (CO) was measured in G4 [with short ischemic time (IT) and immunoadsorption (IA)] after 120 min. Values in control group G2 suddenly decreased after 2 h during hyperacute rejection (HXR), which did not occur in G1 and G3

from both groups at corresponding experimental times. A P value of less than 0.05 was considered a statistically significant difference.

Results

Perfusion time, heart weight increase, and hemodynamic parameters

Total perfusion time of 356 ± 46.1 min in G1 (4 h IT without IA) was significantly prolonged if compared to the control group G2 (short IT and without IA) with 125(31.3 min and was nearly as long as in G3 (IT and IA) with 310 ± 44.2 min and G4 (IA) with 335 ± 37.4 min. Thus IA had no additional impact but was effective by itself in the working heart mode.

In control group 2 (short IT), heart weight increased by 23.9 ± 5.24 %/h which was significantly more than in G1 with long IT (5.67 ± 0.81 %/h). G2 exceeded G4 with IA (5.69 ± 1.43 %/h; $P < 0.05$) and G3 with IT and IA (8.1 ± 1.6 %/h; n.s.). As demonstrated in Fig. 2, the maximum CO was measured in G4 (mean value 338.5 ± 16 ml/min) being nearly constant for the first 4 h. Mean CO was higher if compared to G1 (193.3 ± 2 ml/min; $P < 0.05$), G2 (155 ± 86.8 ml/min; n.s.), and G3 (198.9 ± 15 ml/min; $P < 0.001$). CO decreased suddenly in control group 2 after 2 h due to HXR, which did not occur in G1 and G3.

CF serves as a parameter for micro- and macrovascular thrombosis in HXR. Mean CF was significantly lower in control group 2 (59.4 ± 20.2 ml/min) than in G1 (with IT: 157.9 ± 9.1 ml/min), G3 (IT and IA: 172 ± 11 ml/min), and G4 (with IA: 151.2 ± 11 ml/min). SCF considering heart weight was significantly higher between 120 and 180 min in G1 and G3 with long IT if compared to groups with short IT, especially to G2 with HXR (Fig. 3). SCF tended to reach a maximum with IT and IA (n.s. vs G1).

SWI in the control group G2 decreased significantly after 2 h, whereas this phenomenon could not be found in G1 and G3 with a long IT until 6 h of perfusion (Fig. 4). SWI in G4 (with IA and short IT) was improved later than 6 h of perfusion if compared to the other groups.

Serology

Two cycles of Ig-Therasorb column treatment eliminated 84 % IgG, from 10 to less than 2 mg/dl (Fig. 5 a). In untreated groups G1 and G2, immunoglobulin levels remained constantly high in both groups. IgM was removed by IA by 83 % in G3 and G4 (Fig. 5 b). In the untreated group with 4 h IT, the levels of IgM in the perfusate were reduced by half after the start of reperfusion. In G2, IgM antibodies rebounded after 120 min. IgA was removed by IA in G3 (IT and IA) from 1.56 ± 0.14 to 0.25 ± 0.06 mg/dl, in G4 (IA) from 2.47 ± 0.38 to 0.52 ± 0.23 mg/dl, and in G1 from 2.03 ± 0.43 to 1.32 ± 0.24 mg/dl (Fig. 5 c).

Elimination of anti-pig antibodies from plasma after 4 h IT in G1 and G3 was at least as effective as in G4 with IA treatment only. In G2 (without IA and short IT) the graft removed the majority of XNAb after 30 min of xenoperfusion (Fig. 5 d). Concerning serological parameters of myocardial damage there was only a significant difference with regard to CK (Fig. 6 a), CK-MB, GOT (Fig. 6 b), and LDH (Fig. 6 c) between the

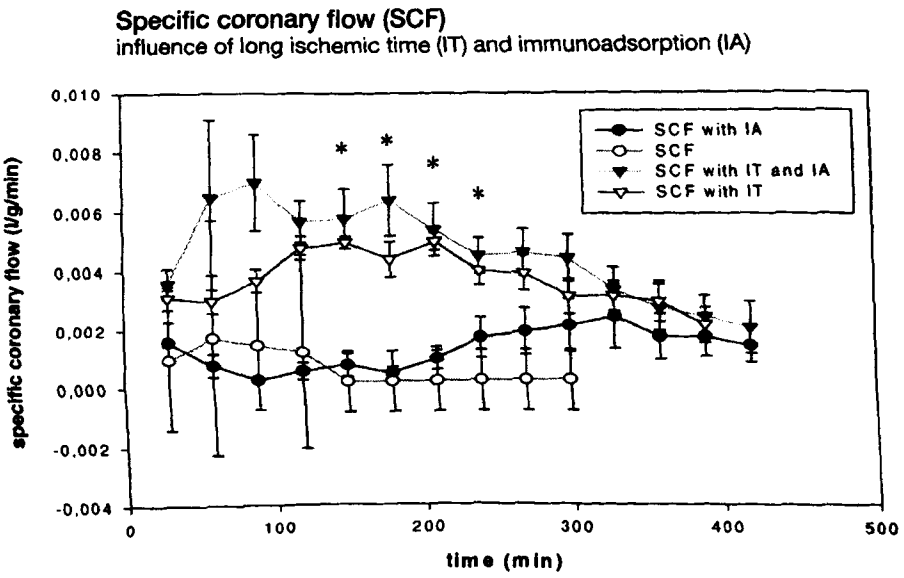


Fig. 3 Specific coronary flow (SCF = cardiac flow ÷ heart weight) was significantly higher between 2 and 3 h of perfusion in groups G1 and G3 with long IT if compared to groups with short IT especially to G2

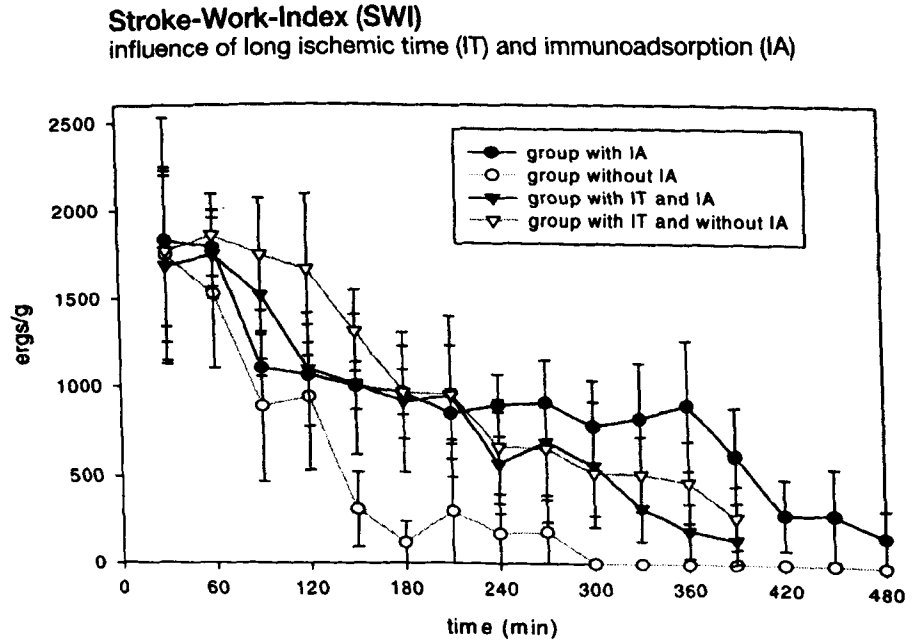
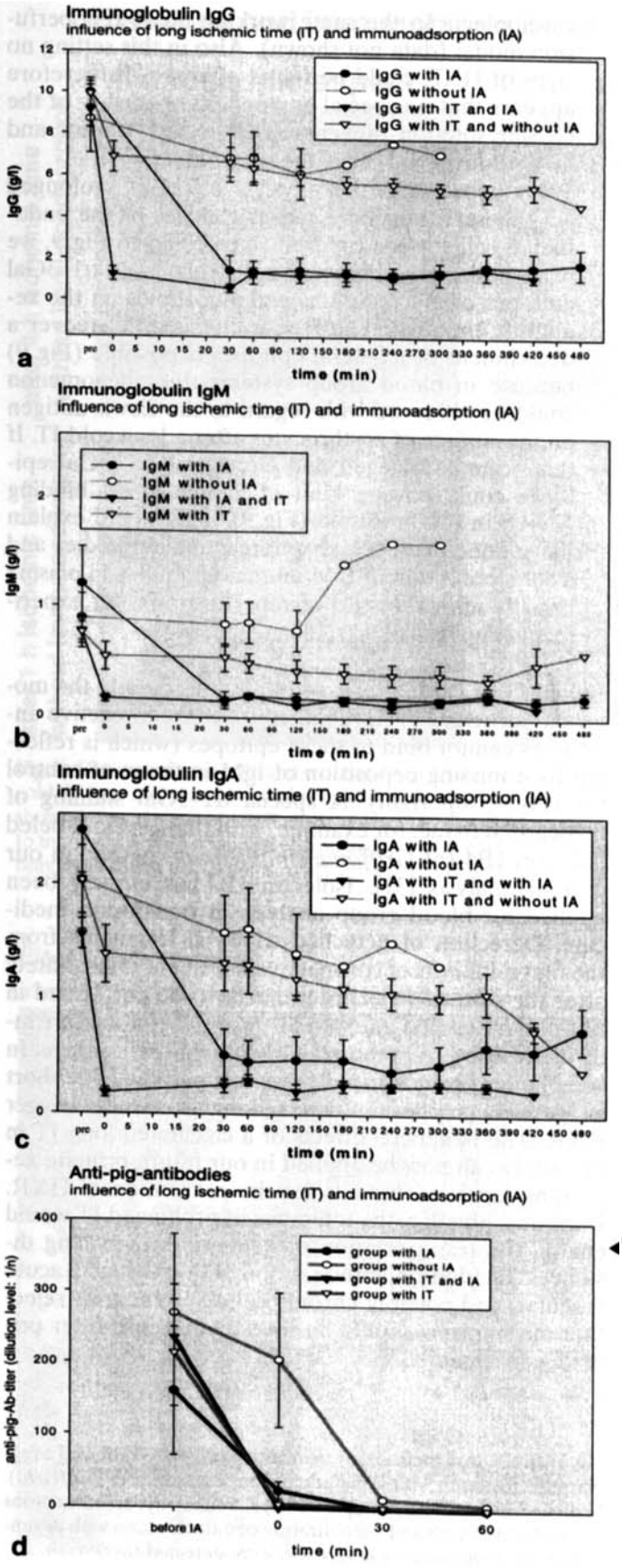


Fig. 4 Stroke-work index [SWI = ((mean arterial pressure–preload) × CO × 1333) ÷ (heart rate × heart weight)] in G2 decreased markedly after 2 h, whereas in G1 and G3 with long IT, SWI decreased slowly until 390 min. The highest SWI was measured in G4 (with IA and short IT), longer than 6 h of xenoperfusion

groups with long IT (G1 and G3) and short IT (G2 and G4). These enzymes increased markedly in groups with long IT, especially without IA, in contrast to the groups with short IT. The level of serological parameters indicating myocardial damage showed the lowest level in groups G2 and G4.

Histology and immunohistochemistry

Hearts treated with short IT but without IA demonstrated massive hemorrhagic changes in contrast to analogous hearts after 4 h cold IT (Fig. 7; *top left*). These hearts of G1 showed a ventricular dilatation, but a nearly inconspicuous myocardium after 6 h of perfusion. Histological examination with hematoxylin and eosin staining revealed typical signs of HXR, such as intravascular thrombosis, edema, focal hemorrhage, and single cell necrosis in G2 only. G1 showed open vessels and an intact myocardial structure, but a slight interstitial edema after 6 h of perfusion (Fig. 7; *top middle*). Immunohistochemistry showed clear IgM antibody deposition in myocardial tissue of G2 (Fig. 7; *bottom right*) which was not detected in the corresponding group with 4 h



IT. Immunohistochemical staining of complement C3 and C4 in myocardial tissue showed a few deposits of the membrane attack complex C5b-9 after 4 h IT in contrast to the IA group G3 with no deposits. The most intense C5b-9 staining was in G2.

Electron microscopic investigation demonstrated in G1 (long IT) a regular ultrastructure with a slight edema, but no signs of HXR (Fig. 8, *right*) after 390 min perfusion time. Exclusively in control group 2 all signs of HXR, such as hypercontractions of contractile bands and vacuolization of mitochondria with degenerative swelling and cristae rupture after 120 min perfusion were found (Fig. 8, *left*). In summary, typical histological signs of HXR (light and electron microscopy, immunohistochemistry) could only be found in G2.

Discussion

These results confirm the suggestion, that a long IT of 4 h, which represents the upper limit for current clinical practice, deteriorates hemodynamic parameters, such as CO and SWI as well as serological parameters (CK, GOT, LDH), which confirms an ischemia/reperfusion injury. Surprisingly, typical signs of HXR, such as a decrease of CF caused by coronary thrombosis, macro- and microscopic tissue damage as well as immunoglobulin and complement deposition, is missing in the group without IA and 4 h IT. Thus a long cold preservation time showed beneficial effects in this model of discordant xenogeneic heart transplantation. This is in contrast to non-transplant studies supposing that ischemia and reperfusion of endothelial cells may activate complement by expression of neoantigens on the endothelial cell surface [30].

Detailed analyses are necessary to prove two hypotheses:

1. The results could be attributed to specific effects of the cardioplegic solution (Celsior). In a recent study we used University of Wisconsin solution (UW) for

Fig. 5 **a** IA (2 cycles) removed 84% IgG immunoglobulins in G3 and G4. In untreated groups G1 and G2 immunoglobulin levels were constantly high in both groups. **b** IA eliminated 83% immunoglobulin IgM in G3 and G4. In the untreated group with 4 h IT, levels of immunoglobulin in the perfusate were almost half after start of reperfusion. In control group G2, IgM antibodies increased again after 2 h. **c** IA removed 84% IgA in G3 (IT and IA), 81% IgA in G4 (IA), and in G1 (IT) by 35% without IA. **d** Xenoreactive anti-pig antibodies, measured in a hemagglutination assay, were eliminated from plasma at least equally effectively after 4 h IT (G1 and G3) as in G4 with IA treatment only. In the control group G2 (without IA and short IT) xenoreactive natural antibodies (XNAb) were adsorbed on the graft after 30 min of xenogeneic perfusion

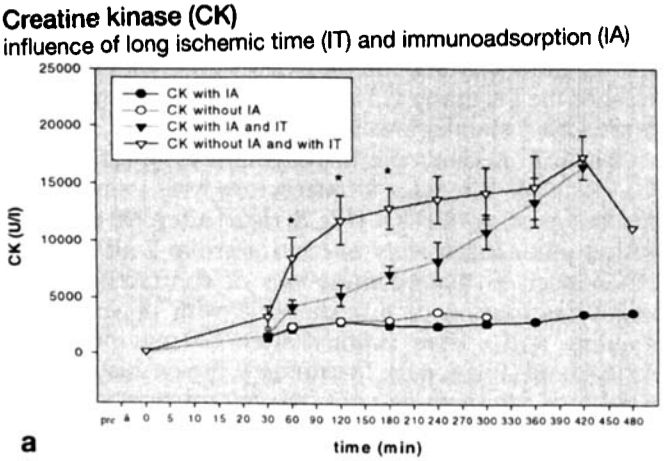


Fig. 6 **a** Creatine kinase (CK) and CK-MB as parameters of myocardial damage showed a difference between groups with and without IA after long IT. They increased markedly in G1 without IA in contrast to groups with short IT. **b** Glutamic oxalacetic transaminase (GOT) demonstrated a significant increase in groups with long IT particularly in G1 without IA. **c** Lactate dehydrogenase levels were similar to CK and GOT. The release was increased in G1 if compared to G3, but not in G2 and G4

cardioplegia in the same working heart xenoperfusion model (data not shown). Also in this setting no signs of HXR could be found after 4 h. It therefore appears that no special component or quality of the Celsior solution prevents HXR except Celsior and UW solution both have the same effect.

2. We suppose, that the physical effect of prolonged 4 °C preservation time causes changes of the endothelial cell surface by itself. According to Fig. 9, we hypothesize that the chemical structure of α1–3Gal epitopes of glycoproteins and glycolipids on the xenograft endothelial surface is changed. Moreover a detachment of α1–3Gal epitopes is possible (Fig. 9) because in blood group systems this phenomenon could be observed with regard to the Lewis antigen on the surface of erythrocytes after a long cold IT. If this occurs, detached and circulating α1–3Gal epitopes could have a kind of hapten effect binding XNAb in the perfusate (Fig. 9). This would explain the sudden decrease of xenoreactive antibodies and a 50% reduction of IgM immunoglobulins in plasma initially after the start of reperfusion in our experiment (Fig. 5b + d).

If cold IT or cardioplegic solution does modify the molecular structure of α1–3Gal epitopes, xenoreactive antibodies cannot bind to these epitopes (which is reflected by a missing deposition of IgM in tissue of control G1 (Fig. 7, *top right*). A special α1–3Gal staining of myocardial tissue, for example, with fluorescein-labeled isolectin 1B4 (of *Griffonia simplicifolia*) is used in our current analysis [31]. Isolectin 1B4 has already been applied for blood group analysis in transfusion medicine. Detection of detached α1–3Gal fragments from the first milliliters of coronary vein effluent immediately after the start of reperfusion needs to be performed in order to prove that after 4 h IT α1–3Gal epitopes are indeed detached from the graft endothelial cell surface. In our previous primate studies we selected the IT as short as possible supposing that a long IT would trigger HXR. The beneficial effects of a calculated long IT in this study can now be applied in our future primate xenotransplantation models for the prevention of HXR. In clinical practice, the tolerance of prolonged IT would enable the transportation of xenografts over long distances. In addition, effects of long IT on delayed, acute vascular, and possibly chronic cellular xenograft rejection mechanisms should be investigated in further primate experiments.

Fig. 8 In electron microscopy myocardial cells of G1 showed a regular ultrastructure with slight edema, but no signs of HXR (*right*). In control group 2, typical signs of HXR, such as hypercontractions of contractile bands and vacuolization of mitochondria with degenerative swelling and cristae rupture, were detected (*left*)

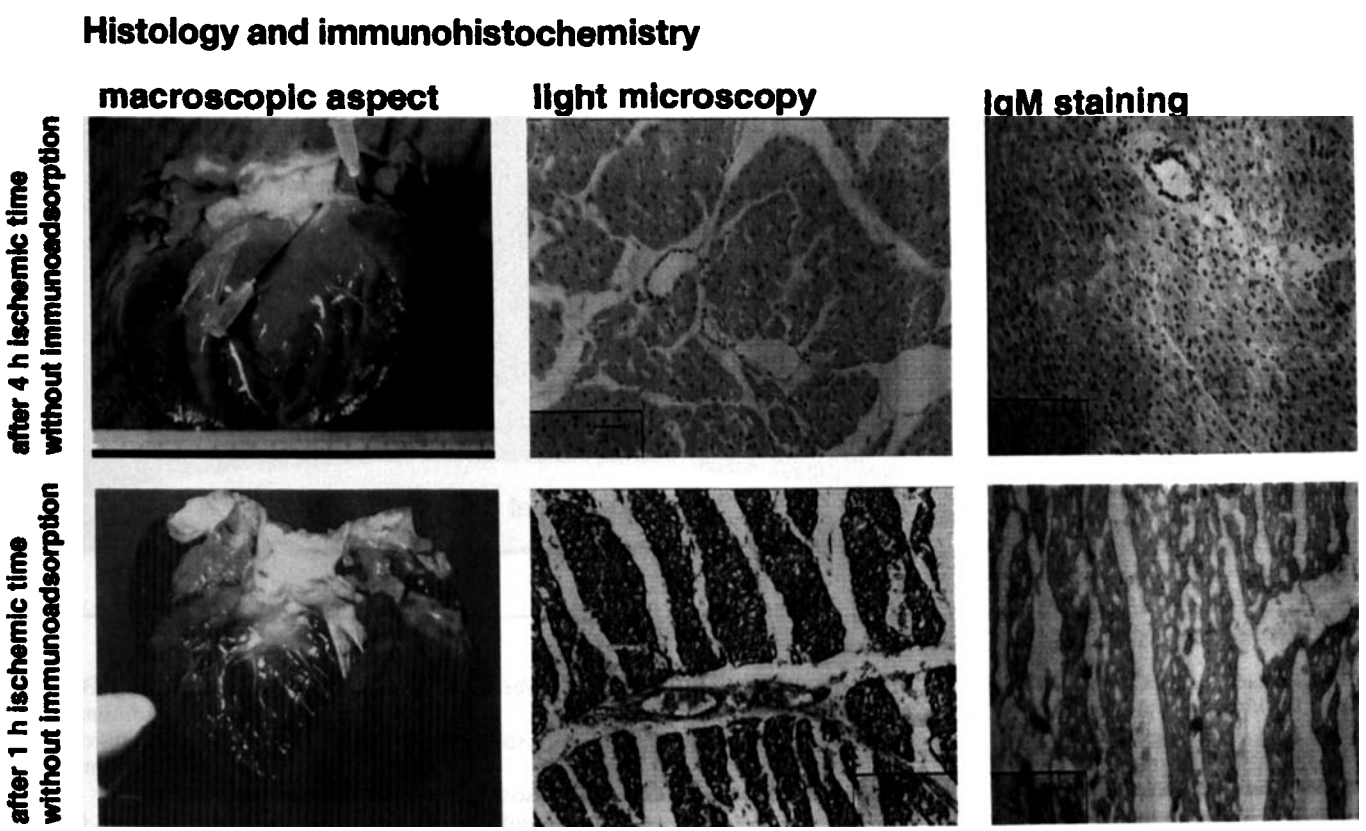
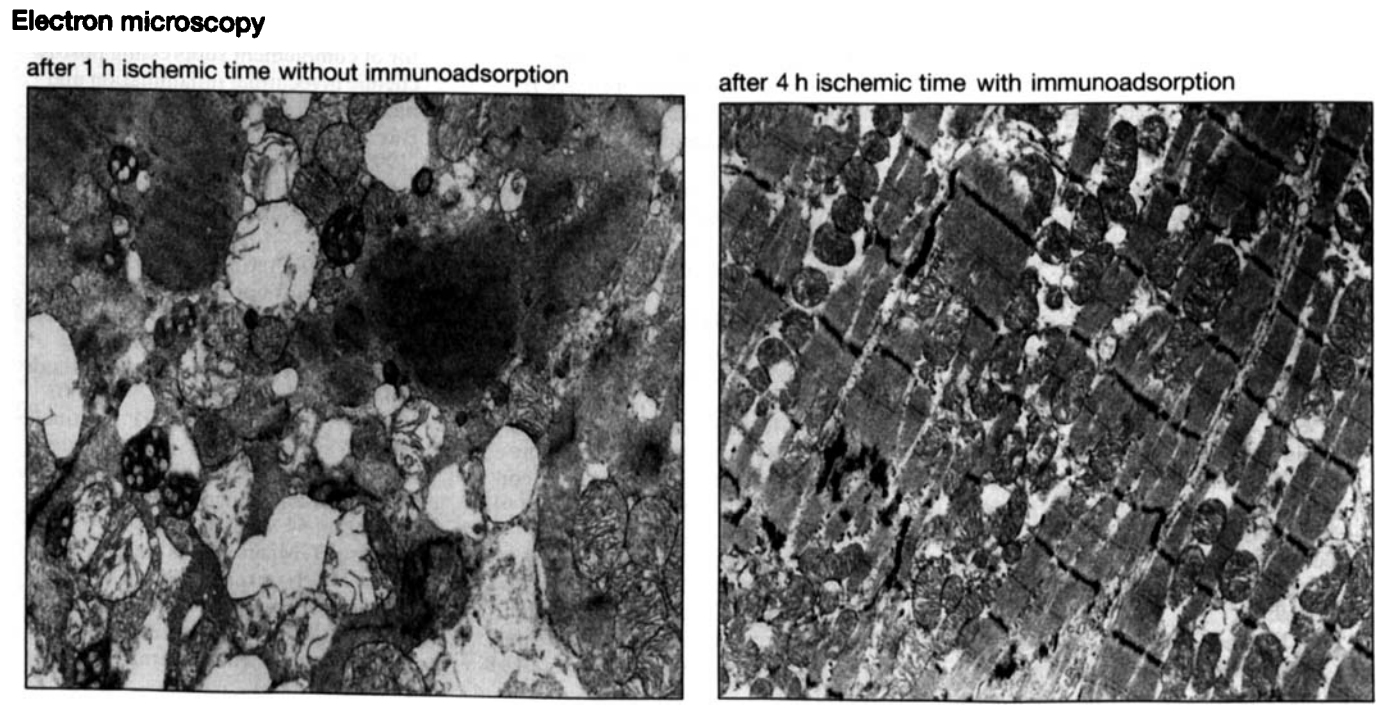


Fig. 7 Hearts with short IT and without IA (G2) demonstrated massive hemorrhagic changes (*bottom left*) in contrast to hearts after 4 h cold IT (*top left*). Histologically (light microscope with hematoxylin and eosin staining), the myocardium of G2 without any treatment showed typical signs of HXR, such as intravascular thrombosis, edema, focal hemorrhage, and cell necrosis (*bottom middle*) in contrast to tissue of G1 with open vessels and an intact myocardial structure (*top middle*). Immunohistochemically, IgM antibody deposition could only be found in G2 (*bottom right*), but not in G1 with 4 h IT (*top right*)



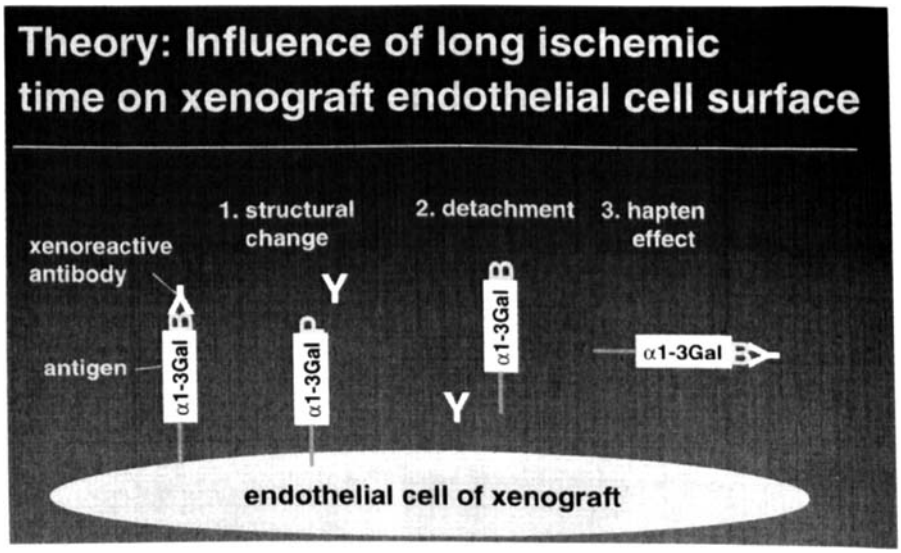


Fig. 9 We suppose that 4 h cold IT causes molecular changes of the glycosylation of α1–3Gal epitopes of glycoproteins and glycolipids on the endothelial cell surface (*1*). The α1–3Gal epitopes can possibly be detached (*2*). These circulating α1–3Gal epitopes could have a kind of hapten effect, thereby binding XNAb immediately after start of reperfusion (*3*)

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