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Heparin and phentolamine combined, rather than heparin alone, improves hepatic microvascular procurement in a non-heart-beating donor rat-model

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Abstract Improvement of organ procurement from non-heart-beating donors (NHBDs) could increase the donor organ pool for liver transplantation. Whether anti-coagulative and anti-vasospastic substances can improve hepatic microvascular preservation from NHBDs is unknown. In donor rats which were pretreated with either heparin ($n = 6$) or heparin combined with phentolamine ($n = 7$) 10 min prior to cardiac arrest, the extent and homogeneity of hepatic microvascular reperfusion was assessed at the end of a 60-min period of cardiac arrest using in situ fluorescence microscopy. Non-pretreated animals with cardiac arrest for 60 min served as controls ($n = 6$). In the non-treated NHBDs, arterial gravity perfusion of 100 cm H_2O with HTK-solution led to a hepatic acinar reperfusion of only ~ 22 % with a remarkably diminished sinusoidal density. Application of heparin prior to cardiac arrest resulted in a two-fold, but insignificant increase of acinar perfusion and sinusoidal density with a still considerable heterogeneity of both parameters. Livers of NHBDs that additionally received phentolamine exhibited significantly increased values of both acinar perfusion and sinusoidal density. Phentolamine was found to reduce heterogeneity of organ microperfusion. Thus, our results indicate that the combined application of heparin and phentolamine is a useful additive for optimizing the quality of organs harvested from NHBDs.

Key words Non-heart-beating donors · Liver · Microvascular preservation · Heparin · Phentolamine · Warm ischemia time

Introduction

In clinical liver transplantation, warm ischemia of liver allografts is the main impediment to hampering the routine use of livers from non-heart-beating donors (NHBDs). Improving the quality of organs harvested from NHBDs could help to alleviate the critical donor shortage by expansion of the donor pool. Beside the ischemic insult, the viability of the donor liver depends on a variety of factors involved in organ retrieval and preservation, such as agonal events in the donor [3], perfusion techniques, perfusion solutions, and preservation methods [11]. Optimal perfusion of the liver in the initial phase of organ preservation must be considered a main determinant of primary graft function both in case of graft procurement from heart-beating, but in particular from NHBDs. Therefore, investigations of ways of achieving sufficient microvascular preservation are required to establish the safety and efficacy of liver transplantation from NHBDs.

In accordance with the Pittsburgh protocol for organ procurement, the two so far existing clinical studies on liver transplantation from NHBDs administer heparin prior to cardiac arrest [1, 4] despite the lack of studies showing the effectivity of and the need for heparin in liver procurement from NHBDs. Besides heparin,

D'Alessandro and colleagues [4] applied phentolamine prior to cardiac arrest to prevent agonal arterial vasospasm [3]. Whether alpha-adrenergic antagonists like phentolamine have any beneficial effect on liver preservation with NHBDs is not known. The empirical use of these substances simply reflects the fact that optimal methods for organ procurement have still not been defined. This study therefore analyzed the effect of heparin alone and in combination with phentolamine on the perfusion of livers harvested from NHBDs after 60 min of cardiac arrest.

Materials and methods

Animal model

In accordance with the German legislation on protection of animals and the "Guide for the Care and Use of Laboratory Animals (NIH publication no. 86–23, revised 1996), Sprague Dawley rats of either sex (Charles River, Fa. Wiga, Sulzfeld, Germany) with body weights from 250–300 g were used for the experiments. Under pentobarbital anesthesia (50 mg/kg ip) the immediate cardiac arrest was induced by phrenotomy ($n = 19$). In 13 of the 19 animals, heparin (300 U/kg) ($n = 6$) or heparin (300 U/kg) and phentolamine (1 mg/kg) ($n = 7$) were injected via the penile vein 10 min prior to cardiac arrest. Non-pretreated animals with cardiac arrest served as controls ($n = 6$). All livers were subjected to in situ warm ischemia for 60 min. During the period of warm ischemia, the animals were laparotomized, and the abdominal aorta was surgically exposed for insertion of a 14G cannula. The rats were then positioned on their left side and the livers were prepared for in situ fluorescence microscopy by placing the left lobe on a plasticine disk held by an adjustable stage that was attached to the operating pad. By doing so, the lower surface of the liver was situated horizontally to the microscope, which guaranteed an adequately even level of focus for microscopy of the surface of the liver. The exposed area of the left liver lobe was immediately covered with a transparent Saran wrap to prevent drying of tissue [12, 13].

After the 60-min period of cardiac arrest, livers were perfused via the arterial catheter with 100 cc 4 °C cold histidine-tryptophane-ketoglutarate (HTK)-solution by 100 cm H_2O gravity. HTK-solution was supplemented with 0.5 ml 5% fluorescein isothiocyanate (FITC)-dextran 150,000 for visualization and contrast enhancement of the hepatic microcirculation. After the microscopic procedure, the livers were excised, stored in HTK-solution at 4 °C for 24 h, and then rinsed with 10 ml of saline solution for spectrophotometric determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the perfusate.

Fluorescence microscopy and microcirculatory analysis

Microscopy was performed using a modified Zeiss Axio-Tech microscope (Zeiss, Oberkochen, Germany) and epi-illumination technique with a 100 watt mercury lamp. Microscopic images were registered using a charge-coupled device video camera (FK 6990; Prospective Measurements Inc., San Diego, CA, USA) and were transferred to a video system (VO-5800 PS; Sony, Munich, Germany). Using different objectives (× 4/0.16 and × 10/0.30, Zeiss) magnifications of × 140 and × 350 were achieved on the video screen (PVM-1442 QM, diagonal: 330 mm, Sony). By means of a blue filter (excitation/emission wavelengths 450–490 nm/ > 520 nm; Zeiss), the hepatic microvasculature could be visualized upon inflow of FITC-dextran-substituted HTK-solution by immediate bright staining of sinusoids and negative contrast of the parenchymal tissue. Microscopy was performed over the whole period of gravity perfusion with analysis of acinar perfusion (× 4 objective) and sinusoidal density (× 10 objective) by scanning the liver surface meandrically.

Quantitative analysis of the videotapes was performed off-line by frame-to-frame analysis. Sinusoidal density was determined by counting the number of HTK-solution perfused sinusoids crossing a 200 μm raster line [13]. Acinar perfusion was assessed quantitatively by planimetric analysis of the perfused area of liver tissue using a computer-assisted image analysis system (CapImage, Zeintl, Heidelberg, Germany). Acinar perfusion is given in percent of the whole area analyzed. Calculation of the coefficient of variance (standard deviation/mean) of both the microvascular perfusion and the sinusoidal density served as a measure of heterogeneity.

Statistics

All values are expressed as means ± SEM. After proving the assumption of normality and homogeneity of variance across groups, differences between groups were tested by analysis of variance (ANOVA) followed by the appropriate post hoc comparison test (SigmaStat, Jandel Corporation, San Rafael, CA, USA). Significance was defined as $P < 0.05$.

Results

The livers of all animals could be successfully perfused and stored. The livers of animals pretreated with heparin prior to cardiac arrest exhibited the same average time for complete arterial inflow of 100 cc HTK-solution (5.09 ± 0.52 min) as untreated NHBD controls (4.69 ± 1.11 min). Compared to the animals treated with heparin alone, however, the livers of heparin/phentolamine pretreated animals exhibited a shorter perfusion time (3.63 ± 0.45 min, $P = 0.056$).

Livers flushed with HTK-solution after 60 min of cardiac arrest exhibited marked acinar perfusion failure (22 ± 5%) with a drastic reduction of the number of perfused sinusoids (1.4 ± 0.2 sinusoids/200 μm) (Figs. 1 and 2). Intravenous application of heparin 10 min prior to cardiac arrest resulted in a two-fold, but insignificant increase of both microvascular perfusion (50 ± 15%) and sinusoidal density (2.9 ± 0.8 perfused sinusoids/200 μm) (Figs. 1 and 2). Despite the overall increase of the hepatic microperfusion upon heparin pretreatment of NHBDs, analysis of the coefficients of variance for acinar perfusion and sinusoidal density revealed mean values comparable to that of non-treated NHBD livers with considerably high standard errors, indicating still a substantial spatial heterogeneity in organ perfusion (Figs. 3 and 4). In contrast, the additional application of phentolamine prior to cardiac arrest significantly improved acinar perfusion and sinusoidal density upon ar-

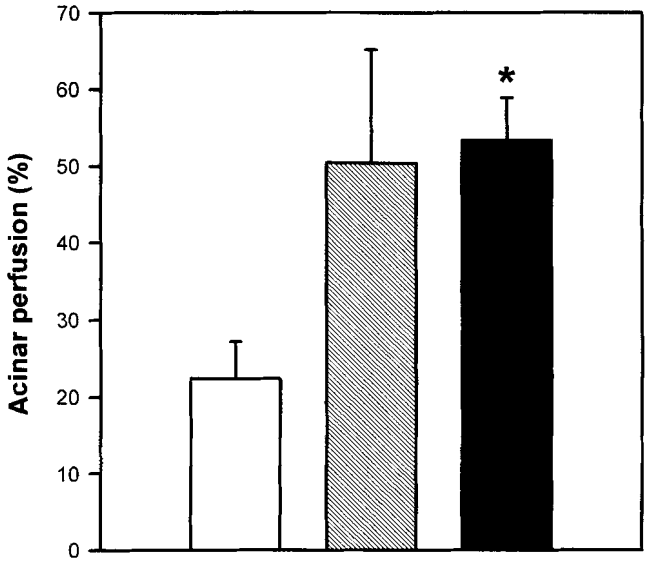

Fig. 1 Acinar perfusion (%) of livers from NHBD rats after 60 min of cardiac arrest (warm ischemia). 10 min prior to cardiac arrest either heparin (300 U/kg) ($n = 6$; *crosshatched bars*) or heparin (300 U/kg) and phentolamine (1 mg/kg) ($n = 7$; *filled bars*) were injected via the penile vein. Non-pretreated animals with cardiac arrest served as controls ($n = 6$; *open bars*). Using in situ fluorescence microscopy, livers were analyzed during exclusive arterial perfusion (100 cm H_2O gravity pressure). Mean ± SEM; $^*P < 0.05$ vs control

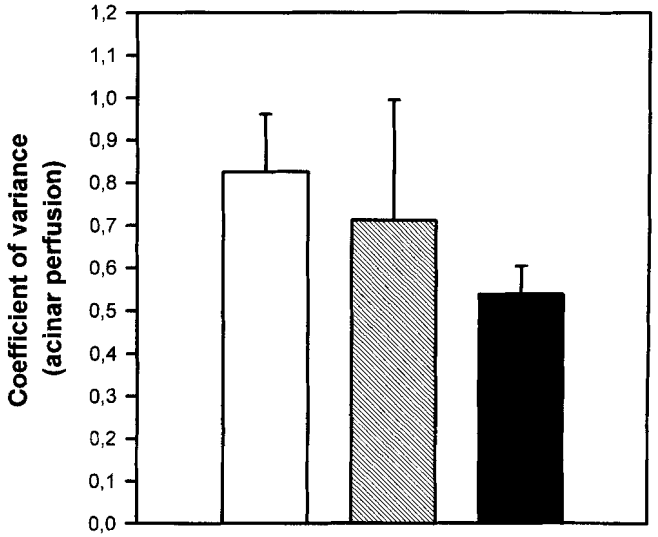

Fig. 3 Coefficient of variance (standard deviation/mean) of acinar perfusion of livers from NHBD rats after 60 min of cardiac arrest (warm ischemia). 10 min prior to cardiac arrest either heparin (300 U/kg) ($n = 6$; *crosshatched bars*) or heparin (300 U/kg) and phentolamine (1 mg/kg) ($n = 7$; *filled bars*) were injected via the penile vein. Non-pretreated animals with cardiac arrest served as controls ($n = 6$; *open bars*). Using in situ fluorescence microscopy, livers were analyzed during exclusive arterial perfusion (100 cm H_2O gravity pressure). Mean ± SEM

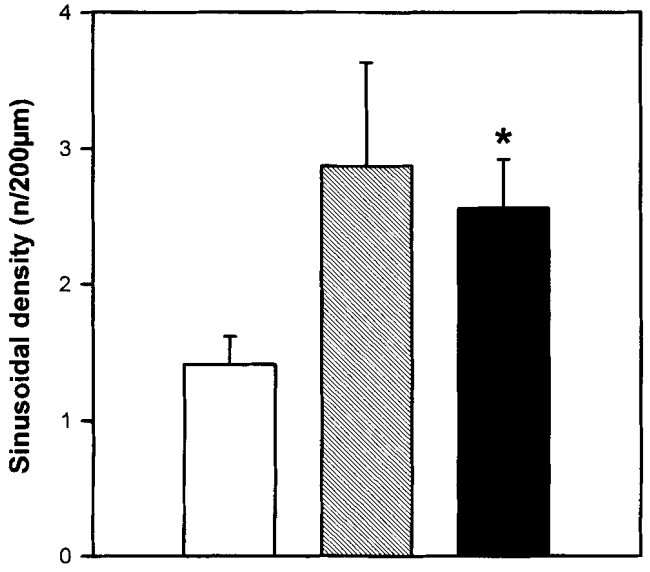

Fig. 2 Sinusoidal density (number of perfused sinusoids per 200 µm) of livers from NHBD rats after 60 min of cardiac arrest (warm ischemia). 10 min prior to cardiac arrest either heparin (300 U/kg) ($n = 6$; *crosshatched bars*) or heparin (300 U/kg) and phentolamine (1 mg/kg) ($n = 7$; *filled bars*) were injected via the penile vein. Non-pretreated animals with cardiac arrest served as controls ($n = 6$; *open bars*). Using in situ fluorescence microscopy, livers were analyzed during exclusive arterial perfusion (100 cm H_2O gravity pressure). Mean ± SEM; $^*P < 0.05$ vs control

terial flushing after 60 min of warm ischemia (Figs. 1 and 2), and caused a remarkable reduction of the spatial heterogeneity of sinusoidal perfusion (Fig. 4).

Surprisingly, liver enzyme determination revealed the highest activities in the 24-h perfusate in the heparin and phentolamine-pretreated group, whereas liver enzymes acivities were significantly and comparably lower both in the heparin-pretreated and in the untreated NHBD group (Table 1).

Discussion

During the last decade, clinical and experimental studies have demonstrated that liver harvesting from NHBDs may greatly contribute to the expansion of human liver pool [1, 4, 6, 7, 10, 15]. Despite this, there are still no definitive recommendations for the use of drugs potentially improving the quality of organ preservation. The present study shows for the first time that the combined use of heparin and phentolamine improves microvascular organ preservation from NHBDs. Based on these findings, we firmly believe that anticoagulative substances and alpha-adrenergic antagonists, such as heparin and phentolamine are indicated in organ procurement.

The causes of dysfunction or primary nonfunction after hepatic transplantation are related to a variety of

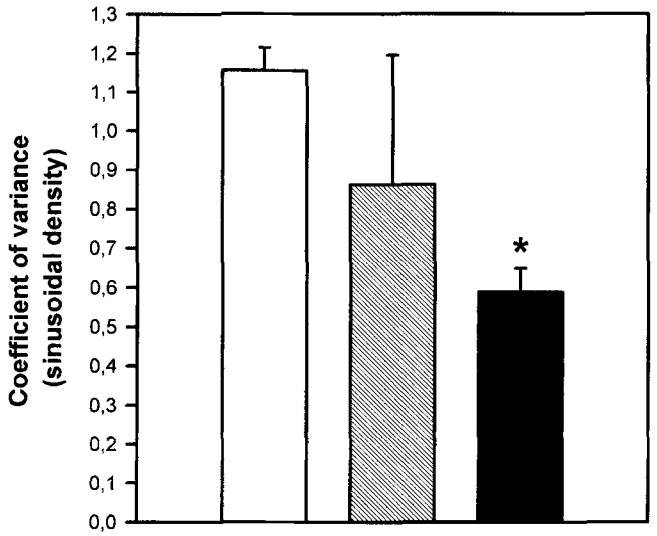

Fig. 4 Coefficient of variance (standard deviation/mean) of sinusoidal density of livers from NHBD rats after 60 min of cardiac arrest (warm ischemia). 10 min prior to cardiac arrest either heparin (300 U/kg) ($n = 6$; *crosshatched bars*) or heparin (300 U/kg) and phentolamine (1 mg/kg) ($n = 7$; *filled bars*) were injected via the penile vein. Non-pretreated animals with cardiac arrest served as controls ($n = 6$; *open bars*). Using in situ fluorescence microscopy, livers were analyzed during exclusive arterial perfusion (100 cm H_2O gravity pressure). Mean ± SEM; $^*P < 0.05$ vs control

factors, including cold storage time and reperfusion injury, immunologic events, as well as graft preservation and primary organ quality. In the case of livers harvested from NHBDs, the suitability of the organs is particularly difficult to define, because of the complexity of factors influencing organ retrieval. Prolonged periods of cardiac arrest and standstill of perfusion drastically impede the uniform flush-out of all blood cells pooling and clotting within the hepatic microvascular segments. In accordance with studies showing that heparin maintains microvascular patency in the liver during and after the low-flow shock state [8, 14], we could observe a two-fold increase in the wash-out of livers after 60 min of cardiac arrest when the donor was pretreated with heparin. Whether this protection is the direct result of the anticoagulative properties of heparin or rather due to the direct effect of the negatively charged nature of heparin cannot be elucidated by the results of the present study. However, there is some evidence that heparin's high electronegative charge keeps erythrocytes and, perhaps, all other blood cells dispersed [9], probably also during the standstill of perfusion. This effect might even be enhanced by an increase in the negative charge of the endothelium as a result of absorption of heparin [2]. Heparin's additional capability of decreasing blood viscosity [2] may further improve patency in the hepatic microvasculature following cardiac arrest and arterial flushing.

Table 1 Activities of AST and ALT in 24-h-perfusate of livers harvested after 60 min of cardiac arrest and iv pretreatment with either heparin (300 U/kg; $n = 6$) or heparin (300 U/kg) and phentolamine (1 mg/kg; $n = 7$) 10 min prior to cardiac arrest. Non-pretreated animals with cardiac arrest served as controls ($n = 6$)

	Controls	Heparin	Heparin/phentolamine
AST (U/L)	274 ± 71	314 ± 30	577 ± 143
ALT (U/L)	271 ± 101	364 ± 52	768 ± 151*

Mean ± SEM; $^*P < 0.05$ vs control

As to whether hepatic microvascular protection is also caused by a heparin-induced reduction of circulating norephinephrine levels, as observed in endotoxic shock [5], cannot be deduced from the present results. However, the importance of sympatho-adrenergic substances in the setting of organ harvest from NHBDs is clearly underlined by the results obtained in animals which did not only receive heparin, but were simultaneously pretreated with phentolamine. The combined application of heparin and phentolamine proved to be significantly more efficient in liver preservation of NHBDs than heparin alone. Beside warm ischemia, cardiac arrest upon withdrawal of life support or discontinuation of cardiopulmonary resuscitation may include agonal events in the donor, leading to a vasospasm of organ-supplying arteries and arterioles upon hypoxia-induced release of catecholamines [3]. The hepatic microcirculation might thus exhibit increased vascular resistance and, therefore, reap the benefits of a blockade of the alpha-adrenergic system by application of phentolamine prior to arterial flushing. In accordance with the effectivity of donor phentolamine-pretreatment in a heparinized porcine asphyxia model [3], we highlight in the present study the capability of phentolamine to reduce particularly the heterogeneity of organ microvascular perfusion, which allows improved preservation conditions and, finally, better graft function. The unexpected higher activity of liver enzymes after 24 h in heparin/phentolamine-pretreated donors therefore may simply reflect the better and more complete 'wash-out' of livers and does not necessarily serve as an indicator of increased liver damage.

In summary, we emphasize the benefit of the combined usage of heparin and phentolamine for adequate liver procurement of NHBD, although we are aware that appropriate microvascular perfusion preservation does not necessarily guarantee final graft viability and survival.

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