Graft preservation with heparinized blood/saline solution induces severe graft dysfunction

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Abstract

OBJECTIVES: Vascular grafts are often stored in cold physiological saline/heparinized blood preservation solution. Until now, only in vitro studies investigated the effect of the aforementioned preservation solutions on endothelial function. The main goal of our study was to compare the storage effect of physiological saline and heparinized blood after short-time cold storage and warm reperfusion in a rat model of aortic transplantation.

METHODS: Aortic abdominal transplantations (n = 6–8/group) were performed in Lewis rats. The donor aortic arches were placed in cold physiological saline and heparinized blood solutions and stored for 2 h. After the 2 h ischaemia, the aortic arches were transplanted into the abdominal aorta of the recipient. Two, 24 h or 1 week after transplantation, implanted grafts were harvested. Endothelium-dependent (acetylcholine) and -independent (sodium nitroprusside) vasorelaxation were investigated in organ bath experiments. DNA strand breaks were assessed by transferase-mediated dUTP nick-end labelling-method and mRNA expression by quantitative real-time polymerase chain reaction. In addition, the expression of CD-31 was also investigated by immunochemistry.

RESULTS: Severely impaired endothelial function and integrity of grafts were shown after 2 and 24 h reperfusion in both groups (maximal vasorelaxation control: 94 ± 1%, heparinized blood: 27 ± 4 and 17 ± 3%, saline 34 ± 5% and 28 ± 5%; CD-31 positive area control: 96 ± 1% blood: 38 ± 8% and 41 ± 6%, saline: 35 ± 12% and 41 ± 7%, respectively P < 0.05). After 1 week, endothelial function and integrity were partially recovered (maximal vasorelaxation: heparinized blood: 46 ± 4%, saline: 46 ± 2%, CD-31 positive area blood: 35 ± 4%, saline: 56 ± 5%, P < 0.05). In addition, mRNA levels of Bax, Bcl-2 and caspase-3 were significantly altered and DNA strand breaks were observed.

CONCLUSIONS: Storage with the generally used physiological saline and heparinized blood solutions is unable to protect the endothelium against cold ischaemia and warm reperfusion injury. A similar weak preservation effect was observed.

Keywords: Ischaemia · Reperfusion · Endothelium · Preservation solution

INTRODUCTION

Coronary artery bypass grafting (CABG) is the most common surgical procedure performed on the heart. The prognosis of surgical revascularization depends largely on the long-term patency of bypass grafts, which is determined by several factors: the progress of heart/vascular disease, the run-off and the biological properties of the implanted graft, injuries during surgical preparation and the degree of ischaemia/reperfusion (IR) injury.

Grafts are stored at temperatures between 4°C and room temperature in different preservation solutions: physiological saline, heparinized blood or Custodiol solution. Until now, studies have only been focused on prevention of endothelial damage as a result of surgical preparation [1] and only in vitro studies examined the effect of preservation solutions against IR injury [2–6]. Among the preservation solutions, the heparinized autologous blood solution seemed to be the most suitable solution for storing human arterial and venous grafts and samples, as indicated by both morphological and functional in vitro studies [7–10]. However, previous in vitro investigations on the endothelial effects of heparinized blood solution resulted in conflicting results [7, 9, 11].

We developed a new in vivo model of arterial revascularization to investigate the early and mid-term effect of IR injury on the endothelial cells of the implanted graft. We therefore evaluated in vivo both the functional and molecular aspects of endothelial dysfunction induced by IR injury.

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MATERIALS AND METHODS

Animals

Male Lewis rats (250–330 g, Charles River, Sulzdorf, Germany) were housed in a room at constant temperature of 22 ± 2°C with 12 h light/dark cycles, and were fed a standard laboratory rat diet and water ad libitum. All procedures concerning animals conformed to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). This investigation was reviewed, and approved by the ethical committee for animal experimentation at Semmelweis University and by the Hungarian government authorities.

Heterotopic aortic transplantation

Aortic transplantations were performed in isogeneic Lewis to Lewis rat strain, therefore no organ rejection can be expected. Aortic rings of the control group were prepared from freshly isolated native aortic arches without ischaemic incubation and reperfusion. Control group aortic arches were cut to rings, and freshly mounted for organ bath. Aortic abdominal transplantations were performed in Lewis rats in all other groups to reproduce revascularization bypasses and to preserve their contribution to vascular impairment. The donor aortic arch was harvested, and flushed using cold physiological saline solution or heparinized blood and stored for 80 min in cold solutions (Table 1). After the 80 min ischaemia, systemic anticoagulation was performed in the recipient rat and the aortic arch was heterotopically transplanted by two end-to-end anastomoses into the abdominal aorta of the isogenic recipient (~40 min surgical procedure). The total ischaemic time was altogether 120 min. 2 h, 24 h or 7 days after transplantation, animals were sacrificed and the implanted graft was harvested. Graft segment was cut transversely into 4-mm width rings. Isolated aortic rings were mounted on hooks in individual organ baths (Radnoti Glass Technology, Monrovia, CA, USA).

Experimental groups

The experimental groups were as given in Table 1.

In vitro organ bath experiments

Isolated aortic rings were mounted on stainless steel hooks in individual organ baths, containing 25 ml of Krebs–Henseleit solution at 37°C and aerated with 95% O2 and 5% CO2. Isometric contractions were recorded using isometric force transducers of a myograph (159901A, Radnoti Glass Technology, Monrovia, CA, USA), digitized, stored and displayed with the IOX Software System (EMKA Technologies, Paris, France). The aortic rings (n = 12–16 aortic rings from N = 6–8 animals) were placed under a resting tension of 2 g (found optimal in preliminary experiments [3, 12]), and equilibrated for 60 min. During this period, tension was periodically adjusted to the desired level and the Krebs–Henseleit solution was changed every 30 min. At the beginning of each experiment, maximal contraction forces to potassium chloride (KCl, 80 mM) were determined and aortic rings were washed until the resting tension was again obtained. Aortic preparations were precontracted with an α-adrenergic receptor agonist, phenylephrine (PE, 10^{-6} M) until a stable plateau was reached, and relaxation responses were examined by adding cumulative concentrations of endothelium-dependent dilator acetylcholine (ACh, 10^{-7}–10^{-4} M). For testing relaxing response of smooth muscle cells, a direct nitric oxide donor, sodium nitroprusside (SNP, 10^{-10}–10^{-5} M) was used. Half-maximal response (EC_{50}) values were obtained from individual concentration–response curves by fitting experimental data to a sigmoidal equation using Origin 7.0 (Microcal Software, Northampton, USA). Contractile responses to PE are expressed as percent of the maximal contraction induced by KCl. The sensitivity to vasorelaxants was assessed by pD2 = –log EC_{50} (M), vasorelaxation [and its maximum (R_{max})] is expressed as percent of the contraction induced by PE (10^{-6} M).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling reaction

To detect DNA strand breaks, TUNEL assay was performed. Following the protocol of the commercial kit provided by the manufacturer (Chemicon International, Temecula, CA, USA), rehydrated sections were treated with 20 µg/ml DNase-free Protease K (Sigma-Aldrich, Germany) to retrieve antigenic epitopes, followed by 3% hydrogen peroxide to quench endogenous peroxidase activity. Free 3'-OH termini were labelled with digoxigenin-dUTP for 1 h at 37°C utilizing a terminal deoxynucleotidyl transferase reaction mixture (Chemicon International). Intracorporated digoxigenin-conjugated nucleotides were detected using a horseradish peroxidase conjugated anti-digoxigenin antibody and 3,3'-diaminobenzidine. Dehydrated sections were cleared in xylene, mounted with Permount (Fischer Scientific, Germany) and coverslips were applied. Semiquantitative histomorphological assessment was performed on all of the stained specimens using conventional microscope. Four different fields were pictured with a digital camera at 200× magnification from each section. TUNEL-positive and -negative cell nuclei were counted and

<table>
<thead>
<tr>
<th>Groups (N = number of animals, N = aortic rings)</th>
<th>Ischaemia (80 min storage and 40 warm ischaemia)</th>
<th>Reperfusion</th>
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</thead>
<tbody>
<tr>
<td>(1) Control (n = 8, N = 16)</td>
<td>No storage</td>
<td></td>
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<tr>
<td>(2a) Saline 2 hR (n = 8, N = 16)</td>
<td>Storage in saline</td>
<td>2 h in vivo</td>
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<tr>
<td>(2b) Saline 24 hR (n = 6, N = 12)</td>
<td>Storage in saline</td>
<td>24 h in vivo</td>
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<tr>
<td>(2c) Saline 7 dR (n = 7, N = 14)</td>
<td>Storage in saline</td>
<td>7 days in</td>
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<tr>
<td>(3a) Heparinized blood 2 hR (n = 8, N = 16)</td>
<td>Storage in heparinized blood</td>
<td>vivo</td>
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<tr>
<td>(3b) Heparinized blood 24 hR (n = 6, N = 12)</td>
<td>Storage in heparinized blood</td>
<td>vivo</td>
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<tr>
<td>(3c) Heparinized blood 7 dR (n = 7, N = 14)</td>
<td>Storage in heparinized blood</td>
<td>vivo</td>
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the TUNEL-positive cell nuclei were calculated as percentage of the
total cell number.

**CD-31 immunohistochemical staining**

To detect the loss of endothelial cells in the lumen of the arterial
grafts, following the manufacturer’s protocol CD-31 staining was
performed (anti-CD31 mouse IgG, Santa Cruz Biotechnology, Inc.,
Heidelberg, Germany). The endothelium-covered area was measured
by the Cell* A imaging software (Olympus, Hamburg, Germany).
During confocal analysis, the vessel was imaged. Expression of
CD-31 was assessed microscopically, as a percentage of the endo-
thelial surface showing a positive immunocytochemical reaction.
The evaluation was conducted by an investigator blinded to the ex-
perimental groups.

**Quantitative real-time polymerase chain reaction**

Total RNA was isolated from the chosen arterial rings with RNeasy
Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) after homogen-
ization (Hielscher UP50 h, Hielscher Ultrasonics GmbH, Teltow,
Germany). RNA concentration and purity were determined at 260,
280 and 230 nm wavelength with a spectrophotometer (Thermo
NanoDrop 2000, Wilmington, USA). Reverse transcription was per-
formed with the QuantiTect Reverse Transcription Kit (Qiagen,
Hilden, Germany) using 400 μg RNA in a volume of 20 μl. Efficiency
of the polymerase chain reaction (PCR) reaction was confirmed
with the standard curve analysis. Every sample was quantified in
duplicate, normalized to glyceraldehyde-3-phosphate dehydrogen-
ase expression. Quantitative real-time PCR was performed on the
LightCycler480 system with the LightCycler480 Probes Master and
Universal ProbeLibrary probes (Roche, Mannheim, Germany).
Relative expressions of bax, bcl-2 and caspase-3 were determined
by qRT-PCR.

**Chemical reagents**

Sodium-phenobarbital (Merial GmbH, Hallbergmoos, Germany)
was used. PE, Ach and SNP were obtained from Sigma-Aldrich
(Taufkirchen, Germany).

**Statistical analysis**

All data are presented as means ± standard error of mean (SEM).
Data were tested for normal distribution with Shapiro–Wilk and
where they met the requirements for parametric analysis (organ
bath experiments), means were tested by analysis of variance and
Bonferroni correction test. For the analysis of PCR result, Kruskal–
Wallis test for multiple comparison was used. A P-value of <0.05
was considered statistically significant.

**RESULTS**

**Vasomotor function**

In aortic rings precontracted with PE, ACh induced a concentra-
tion-dependent relaxation (Fig. 1). After short ischaemia and 2 h
of warm reperfusion, a significant impairment of endothelial func-
tion of arterial rings was demonstrated in blood stored groups
when compared with the control. The endothelial dysfunction

**Figure 1**: Vasomotor function of rat aortic rings. (A) Acetylcholine-induced endothelium-dependent vasorelaxation are shown after 2 h in vivo reperfusion (n = 16 rings/group); (B) acetylcholine-induced endothelium-dependent vasorelaxation is shown after 24 h in vivo reperfusion (n = 12 rings/group); (C) acetylcholine-induced endothelium-dependent vasorelaxation is shown after 7 days in vivo reperfusion (n = 14 rings/group). Values represent mean ± SEM; *P < 0.05 vs control.
was indicated by the reduced maximal relaxation of coronary rings to ACh and the rightward shift of the concentration-response curve and a reduced pD2 to ACh when compared with the control (Fig. 1A). After 24 h of warm reperfusion, the endothelial function worsened compared with the 2 h reperfusion group without reaching the level of statistical significance (Fig. 1B). Partially recovered endothelial function was observed after 7 days of reperfusion in saline and heparinized blood-stored groups (Fig. 1C). There was no significant difference in Rmax for endothelium-independent vasorelaxation of the aortic rings to SNP between the experimental groups. Contractile responses of aortic segments in saline or heparinized blood significantly increased PE-induced maximum contraction compared with the control group.

**Expression of CD-31**

The inner walls of all aortic segments (control + other groups) were covered with endothelium showing a positive CD-31 reaction. The percentage of stained endothelium differed between the control and saline and heparinized blood-stored groups. In both the control and the saline and heparinized blood-stored groups, there were regions of endothelium that showed no reaction. In the control group, the endothelial surface showing positive reaction for the CD-31 antigen (Fig. 2A) was estimated as 96.3 ± 1.5%, whereas in vessels exposed to cold storage and 2 h warm reperfusion it was 34.9 ± 12.2% in the saline group and 38.7 ± 6.0% in the heparinized blood group. However, after 7 days warm reperfusion, the CD-31 reaction was significantly stronger in the saline group when compared with the control and to the saline group when compared with the control when compared with the control; in the control group, the DNA strand break (Fig. 2B) was estimated as 16 ± 3%, whereas in vessels exposed to cold storage and 2 h warm reperfusion it was 46.9 ± 4.5% in the saline group and 59.5 ± 3.4% in the heparinized blood group (P < 0.05). After 24 h warm reperfusion, DNA strand break was 67.1 ± 5.4% in the saline group and 68.4 ± 2.0% in the heparinized blood group. After 7 days warm reperfusion, TUNEL-positive cells were also significantly altered: 57.8 ± 3.5% in the saline group and 61.1 ± 2.0% in the heparinized blood group.

**mRNA expression following blood/saline storage and in vivo reperfusion**

mRNA expression of Bax was significantly increased after 2 h reperfusion in both groups when compared with the control; however, after 24 h and 7-day reperfusion there was a significant up-regulation in the heparinized blood group when compared with the saline group (Fig. 3A). We also observed a significant up-regulation of gene caspase-3 after 2 h reperfusion in both treatment groups and after 7-day reperfusion in the heparinized blood group when compared with the control (Fig. 3C). mRNA expression of bcl-2 significantly increased after 2 h reperfusion in the saline group when compared with the control and to the blood group. Furthermore, a significantly down-regulation of gene bcl-2 was observed in the saline group after 24 h and 7-day reperfusion when compared with the control and blood groups (Fig. 3B).

**DISCUSSION**

The major findings of this study are as follows: (i) application of the daily used physiological saline or heparinized blood as preservation solutions for arterial grafts is incapable of reducing the endothelial damage after cold storage and warm reperfusion; (ii) heparinized blood and physiological saline solution have similar weak preservation effect on endothelium; (iii) blood containing

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**Table 2:** Values of maximal relaxation (Rmax, %) and pD2 to acetylcholine, to sodium nitroprusside (SNP) and contraction forces induced by phenylephrine (10^-6 M) in aortic rings in control, saline or heparinized blood group (N: number of aortic rings)

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<tr>
<td>Rmax to acetylcholine (%)</td>
<td>94.7 ± 1.0</td>
<td>33.8 ± 5.7*</td>
<td>29.1 ± 4.0*</td>
<td>19.4 ± 3.1*</td>
<td>16.8 ± 2.8*</td>
<td>41.9 ± 2.8*</td>
<td>46.0 ± 3.6***</td>
</tr>
<tr>
<td>pD2 to acetylcholine</td>
<td>7.5 ± 0.1</td>
<td>6.2 ± 0.2*</td>
<td>5.6 ± 0.4*</td>
<td>6.0 ± 0.5*</td>
<td>6.5 ± 0.3*</td>
<td>6.1 ± 0.3*</td>
<td>6.3 ± 0.4*</td>
</tr>
<tr>
<td>Rmax to SNP (%)</td>
<td>100.6 ± 0.2</td>
<td>101.0 ± 1</td>
<td>100.3 ± 0.27</td>
<td>98.4 ± 1.7</td>
<td>94.7 ± 2.2</td>
<td>103.5 ± 2.3</td>
<td>98.96 ± 5.1</td>
</tr>
<tr>
<td>pD2 to SNP</td>
<td>83.5 ± 0.1</td>
<td>7.7 ± 0.2</td>
<td>8.0 ± 0.2</td>
<td>6.8 ± 0.2**</td>
<td>7.0 ± 0.1***</td>
<td>6.7 ± 0.3***</td>
<td>6.1 ± 0.1**</td>
</tr>
<tr>
<td>Phenylephrine (% of KCl)</td>
<td>85.5 ± 3.34</td>
<td>117.1 ± 3.2*</td>
<td>127.7 ± 2.7*</td>
<td>114.6 ± 5.9</td>
<td>149.0 ± 12.2*</td>
<td>181.7 ± 9.3*</td>
<td>149.6 ± 12.9*</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM.  
*P < 0.05 vs control.  
**P < 0.05 vs saline 2 hR.  
***P < 0.05 vs saline 24 hR.  
****P < 0.05 vs blood 24 hR.
preservation solutions had a negative impact on the endothelium when compared with saline preservation solution.

Neutrophil granulocytes play an important role in the pathogenesis of endothelial ischaemia-reperfusion injury by inducing an inflammatory response with generation of reactive oxygen species (ROS) and release of proteases and proinflammatory cytokines. Several experimental studies reported that after cold storage and warm reperfusion, high levels of ROS severely impair endothelial function, thus leading to vascular dysfunction [13–15]. Damaged endothelial cells are responsible for impaired vasodilatory graft function and for developing vasculopathy [16]. Vascular tone (endothelium-dependent vasorelaxation) of the graft is particularly important in both cardiac and vascular surgeries, as it determines postoperative blood flow and may be responsible for vasospasm, early and late graft thrombosis and stenosis [17].

Until now, experimental and clinical studies have only been focused on how to prevent endothelial damage resulting from surgical preparation [1] and only limited data are available on the effect of preservation solutions against IR injury on endothelium [18]. In our previous work, we investigated the effect of extracorporeal circulation and cold cardioplectic arrest on cardiac and endothelial function in canine model of cardiopulmonary bypass (CPB), which leads to global IR injury [19, 20]. We demonstrated a slightly impaired coronary endothelial function after CPB both in vivo coronary blood flow measurements and by in vitro vascular reactivity experiments [19, 21]. Schroder et al. in an in vitro study showed impaired rat coronary vasodilatory capacity after 8 h of cold storage in Custodiol [22]. However, successful endothelial preservation after a longer ischaemic period was also reported by in vitro examination [23]. Interestingly, Radovits et al. compared the degree of injury on endothelial function caused by in vitro or in vivo hypoxia-reoxygenation (H/R), and concluded that endothelial injury occurring in vessel rings during in vitro H/R is too moderate (probably due to the lack of activated leucocytes) and could not be demonstrated in functional measurements [24]. Therefore, the experimental model of in vitro vascular H/R is not suited for reliable investigation of pharmacological attempts. To simulate the whole process of the reperfusion injury in arterial graft, we designed a new arterial revascularization model in rat.

To the best of our knowledge, this is the first study investigating preservation effects of heparinized blood and saline solutions on the endothelial function of arterial grafts in a clinically relevant model of arterial revascularization. Previous in vitro investigations on the endothelial effects of heparinized blood solution resulted in conflicting results. Tatoulis et al. [11] found increased vasoconstrictor reactivity of radial grafts stored in heparinized blood compared with artery stored in saline. Wilbring et al. showed also a better preserved vascular function of saphenous grafts stored in heparinized blood, and concluded that saline is no longer recommended for intraoperative storage of saphenous grafts [9]. Contrasting these findings, Chong et al. [7] reported significantly enhanced endothelium-dependent vasorelaxation to ACh with radial arteries stored in heparinized blood compared with arteries stored in saline solution.

In our study, we could not demonstrate an improved preservation effect on vasomotor function of heparinized blood solution.
The peak of the endothelial damage in both groups occurred after 24 h of reperfusion as demonstrated by severely damaged endothelium-dependent vasorelaxation, apoptosis rate of TUNEL-staining and increased mRNA expression of Bax and caspase-3 and the decreased levels of Bcl-2 gene expression. These data correlate to our previous investigation (under review) and to the clinical findings of early complications in bypass grafts, as Lockerman et al. found transient ST segment elevation occurring in the first 12–24 h after CABG [25]. It should be noted that the severity of the endothelial dysfunction caused by in vivo reperfusion in our model was more detrimental than in the previous in vitro induced ischaemic injury alone or in the additional hypochlorite-induced reperfusion injury studies [2, 3, 6, 24].

Endothelial integrity and structure (as measured by lower expression of CD-31 on the endothelial surface in the aortic segments and enhanced DNA stand breaks) were also damaged in our experiment. However, we also demonstrated that the endothelial layer of the graft partially recovered after 1 week of reperfusion (Fig. 2), which is in agreement with previous experimental studies reporting that the endothelial cells could regain their functional integrity within a few days after mechanical injury. The endothelial stunning in our model was also demonstrated through the dynamic changes of several genes over time (Fig. 3). However, the significantly stronger structural damage of the endothelium in the heparinized blood group, demonstrated by CD-31 staining and changes of gene expressions (bax, bcl-2 and caspase-3), indicated that blood containing preservation solutions had a negative impact on the endothelium probably through its high level of activated leucocytes.

There are some important clinical implications of this study. Current preservation protocol for the graft is only directed at minimizing mechanical damage during harvesting and the construction of the anastomosis. In the present study, we clearly demonstrated that the saline and heparinized blood solutions are incapable of preserving the structure and integrity of the implanted arterial graft after short-term storage and warm reperfusion.

**Study limitations**

The rat model of aortic transplantation was selected to be a suitable model to evaluate in vivo IR injury. However, this model has certain limitations. Rat aortic tissue differs from arterial grafts for CABG in the structure of the vessel wall, which limits the transferability of the results.

**Conflict of interest:** none declared.

**REFERENCES**


