Is internal thoracic artery resistant to reperfusion injury? Evaluation of the storage of free internal thoracic artery grafts

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ABSTRACT

Objectives: The in situ internal thoracic artery (ITA) is recognized as the best conduit for coronary artery bypass surgery. The ITA—if it is used as an in situ graft—has a much higher late patency rate than any other arterial graft, including a free ITA graft. We sought to determine if the use of the ITA as an in situ/free graft and its storage in preservation solutions, have an effect on endothelial function.

Methods: The ITA was harvested as either a free or in situ graft in a porcine model. Free grafts were stored in different preservation solutions (saline, Custodiol and Tiprotec [both Köhler Chemie GmbH, Bensheim, Germany]). The ITA was anastomosed off pump to the left anterior descending artery (as in situ/free graft). Freshly harvested ITA served as a control. After 2 hours of reperfusion, the implanted grafts were harvested. The assessment of endothelial function, histopathological analysis, and gene expression were performed.

Results: Endothelial function and integrity were severely impaired after reperfusion in the free ITA groups, however, it was partially preserved in the Tiprotec group. Reperfusion injury resulted in increased nitro-oxidative stress, DNA breakage, vascular cell adhesion protein 1, intercellular adhesion molecule-1, and caspase-3 scores, and a decreased endothelial nitric oxide synthase score in the free ITA groups. The in situ ITA graft showed no signs of injury. mRNA levels were significantly altered among the groups.

Conclusions: An early, severe endothelial dysfunction of the stored, free ITA as described, could be completely prevented by the use of an in situ ITA graft. Tiprotec might be a feasible option for storage of free arterial grafts during coronary artery bypass grafting. (J Thorac Cardiovasc Surg 2018;156:1460-9)

Vasomotor function of the internal thoracic artery.

Central Message

Early, severe endothelial dysfunction of the stored, ITA was described, which might play a negative role on patency rate. An in situ ITA graft showed no signs of injury.

Perspective

Our findings might have important implications in terms of graft survival and results after CABG, and they suggest that the implication of an in situ ITA over stored, free ITA graft might improve the beneficial effect of CABG with bilateral ITA grafting. Avoidance of storage solutions (saline/Custodiol) and preparation of the free arterial grafts similar to the in situ grafts should be considered.

See Editorial Commentary page 1470.

Over the past decade, many efforts have been made to improve coronary artery bypass grafting (CABG) results. Interest has been focused on the extended application of

arterial grafts.¹ The internal thoracic artery (ITA) has the most favorable patency among conduits used for CABG.^{2,3} The biological properties of the endothelium can protect the ITA from vasospasm, atherosclerosis, and neointimal hyperplasia (NIH).⁴ Moreover, compared with

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Scanning this QR code will take you to a procedural video.

Abbreviations and Acronyms

ACh	= acetylcholine
CABG	= coronary artery bypass grafting
eNOS	= endothelial nitric oxide synthase
HIF-1	= hypoxia-inducible factor 1
ICAM-1	= intercellular adhesion molecule-1
IR	= ischemia-reperfusion
ITA	= internal thoracic artery
LAD	= left anterior descending artery
MMP-9	= matrix metalloproteinase-9
NIH	= neointimal hyperplasia
NO	= nitric oxide
PCR	= polymerase chain reaction
PE	= phenylephrine
R _{max}	= maximum vasorelaxation
ROS	= reactive oxygen species
SNP	= sodium nitroprusside
TUNEL	= terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
VCAM-1	= vascular cell adhesion protein 1
VEGF	= vascular permeability factor

other conduits, it shows an increased production of nitric oxide (NO).⁴

After successful long-term results of single ITA grafting to the left anterior descending artery (LAD),^{5,6} many groups suggested that the use of both ITAs (free/in situ) would further improve the outcomes.^{7,8} This kind of expectation is on the basis of a hypothesis that in situ and free ITA grafts have similar biological characteristics such as contractility, vasorelaxation, endothelial function, and anatomical structure. However, the long-term results with free ITA grafts are controversial.⁹⁻¹¹ Regarding the importance of an intact endothelial layer for graft patency, several different storage solutions for the

preservation of free vascular grafts have been tested. However, these studies showed poor preservation capacity of routinely used preservation solutions (saline, Custodiol [Köhler Chemie GmbH, Bensheim, Germany]). A new preservation solution (Tiprotec [Köhler Chemie GmbH]), which is an N-acetyl-histidine buffered solution with iron chelators, dramatically improved endothelium preservation of free arterial grafts.¹² For several years, antioxidant drugs that reduce reactive oxygen species (ROS) levels have been depicted as important therapeutic targets for the treatment of restenosis.¹³ To search for the possible differences among stored, free, and in situ ITA grafts, and to verify whether the endothelial function of the stored, free, and in situ ITA grafts were preserved after CABG, we developed a new off-pump arterial revascularization model in pigs.

METHODS**Animals**

Thirty-eight healthy pigs (25-35 kg) were used in this experiment. All procedures concerning animals were 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 (Publication No 86-23, revised 1996). The investigations were reviewed and approved by the local Ethical Committee for Animal Experimentation. The pigs were randomly assigned to the 5 experimental groups (Figure 1): control (n = 6), ITA as a free graft (storage solution: saline; n = 8), ITA as a free graft (storage solution: Custodiol; n = 8), ITA as a free graft (storage solution: Tiprotec; n = 8), or ITA as an in situ graft (n = 8).

Surgical Procedure

Anesthetized pigs underwent an off pump procedure (Video 1). After median sternotomy and pericardiotomy, all left ITAs were harvested as free/in situ grafts.

In the in situ group, the ITA was harvested as a pedicle. After heparin was administered, the ITA was divided distally, the end was closed with a clip. The in situ ITA grafts were subjected to a 1-hour waiting period before the LAD anastomosis.

In the free ITA groups, the ITA was harvested as a pedicle graft. After heparin was administered, the ITA was divided distally and proximally,

FIGURE 1. Groups and treatments. ITA, Internal thoracic artery; PCR, polymerase chain reaction.

VIDEO 1. Evaluation of free and in situ internal thoracic artery grafts. Surgical procedure and results. Video available at: [https://www.jtcvs.org/article/S0022-5223\(18\)31509-5/fulltext](https://www.jtcvs.org/article/S0022-5223(18)31509-5/fulltext).

then removed and subsequently flushed using cold preservation solution, thereafter it was stored for 1 hour. After 1 hour of ischemia, systemic anticoagulation was performed, and the ITA was anastomosed to the LAD and to the ascending aorta. In the in situ ITA group, the ITA was anastomosed to the LAD. The LAD was closed via ligature proximal to the anastomosis. Coronary blood flow was measured on implanted grafts with a perivascular ultrasonic flow probe (Transonic Systems Inc, Ithaca, NY) to control anastomoses' quality. Control group ITAs were freshly harvested.

After 2 hours of reperfusion (at the end of the experiments), ITA graft samples were taken and immediately immersed in fluid nitrogen (-196°C) and stored frozen at -80°C until the biochemical measurements were performed (nitrotyrosine, caspase-3, intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion protein 1 (VCAM-1), endothelial nitric oxide synthase (eNOS) stainings, and polymerase chain reaction [PCR]). Additionally, further ITA segments from each experimental group were fixed in paraformaldehyde solution (4%) and embedded in paraffin. The 3-mm thick sections were placed on adhesive slides. These paraffin slides were used for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) and CD-31 staining.

In Vitro Organ Bath Experiments

After 2 hours of reperfusion, the implanted grafts were harvested and used for functional isometric tension measurements as described previously.¹² At the beginning of each experiment, maximal contraction forces to KCl (80 mM) were determined and ITA rings were washed until the resting tension was again obtained. ITA preparations were precontracted with an α -adrenergic receptor agonist, phenylephrine (PE; 10^{-6} M), and relaxation responses were examined by adding cumulative concentrations of acetylcholine (ACh; 10^{-9} to 10^{-4} M). For testing the relaxation response of smooth muscle cells, sodium nitroprusside (SNP; 10^{-10} to 10^{-5} M) was used. Each specimen (ITA ring) was tested at 10 increasing concentration levels. Half-maximal response values were obtained from individual concentration response curves by fitting experimental data to a sigmoidal equation using Origin 7.0 (Microcal Software, Northampton, Mass). Contractile responses to PE are expressed as percent of the maximal contraction induced by KCl. The sensitivity to vasorelaxants was assessed using the formula: $\text{pD}_2 = \text{negative value for log half-maximal response (M)}$, vasorelaxation (and its maximum [R_{max}]) is expressed as percent of the contraction induced by PE (10^{-6} M). Only 1 datum (the mean value of 3–4 aortic rings per animal) was used for the statistical analysis.

TUNEL Reaction

We performed TUNEL staining to detect DNA strand breaks as previously described.¹² TUNEL-positive cell nuclei were counted by 2 blinded observers and thus, the mean value of the counted TUNEL-positive cell nuclei was used for the statistical analysis.

TABLE 1. Gene table

Gene symbol	Alias
<i>MMP9</i>	Matrix metalloproteinase 9
<i>NOS3</i>	Endothelial nitric oxide synthase
<i>ICAM-1</i>	Intercellular adhesion molecule-1
<i>SOD1</i>	Superoxide dismutase
<i>HIF1A</i>	Hypoxia-inducible factor 1- α
<i>IL1B1</i>	Interleukin-1 β
<i>IL6</i>	Interleukin-6
<i>TNF</i>	Tumor necrosis factor α
<i>VCAM-1</i>	Vascular cell adhesion protein 1
<i>BAX</i>	Bcl-2-like protein 4
<i>BCL2</i>	B-cell lymphoma 2
<i>BCL2L1</i>	Bcl-2-Like Protein 1
<i>CASP1</i>	Caspase-1
<i>CASP3</i>	Caspase-3
<i>IL10</i>	Interleukin-10
<i>IL7</i>	Interleukin-7
<i>PECAM1</i>	Platelet Endothelial Cell Adhesion Molecule
<i>SELPLG</i>	P-selectin glycoprotein ligand 1
<i>SERPINE1</i>	Plasminogen activator inhibitor 1
<i>VEGFA</i>	Vascular permeability factor
<i>VWF</i>	Von Willebrand factor
<i>CIDEB</i>	Cell-death-inducing DNA-fragmentation-factor-like effector B
<i>CXCL8</i>	Alveolar macrophage chemotactic factor I
<i>JUN</i>	Proto-oncogene c-jun
<i>IL1B2</i>	Interleukin-1 β
<i>CCNA1</i>	Cyclin A1
<i>MYB</i>	V-myb myeloblastosis viral oncogene homolog
<i>TROAP</i>	Trophinin associated protein
<i>CAMK1</i>	Calcium/calmodulin-dependent protein kinase type 1
<i>DLG4</i>	Disks large homolog 4
<i>GRIN2D</i>	Glutamate ionotropic receptor NMDA type subunit 2D
<i>NOS1</i>	nNOS
<i>PPP3CA</i>	Protein phosphatase 3
<i>LOC100522128</i>	PRKAR1B
<i>PRKCA</i>	Protein kinase C α
<i>NOS2</i>	Nitric oxide synthase
<i>DUOX1</i>	Dual oxidase 1
<i>SELL</i>	L-selectin
<i>GAPDH</i>	Peptidyl-cysteine S-nitrosylase
<i>B2M</i>	Beta 2-microglobulin

Immunohistochemical Stainings (CD-31, Nitrotyrosine, Caspase-3, eNOS, VCAM-1, ICAM-1)

To detect the loss of endothelial cells in the lumen of the ITA grafts, CD-31 staining was performed following the manufacturer's protocol

(anti-CD31 porcine immunoglobulin G; Santa Cruz Biotechnology Inc, Heidelberg, Germany). According to the previously described methods,¹⁴ we performed immunohistochemical staining on ITA rings for nitrotyrosine, a marker of peroxynitrite-mediated damage, and for caspase-3, a marker of apoptotic injury. Immunohistological stainings for eNOS, ICAM-1, and VCAM-1 were performed using the immunalkaline phosphatase technique described by Mason and colleagues.¹⁵ Results were performed by 2 or 3 blinded observers and thus, the mean value of these calculations was used for the statistical analysis.

Gene Expression Analysis Using RT² Profiler PCR Arrays (Qiagen, Hilden, Germany)

Using RT² Profiler PCR Arrays, we determined the expression patterns of 38 genes (Table 1). Total RNA and microRNA were extracted from ITA graft samples with a miRNeasy Mini Kit (Qiagen, Hilden, Germany) and were reverse-transcribed into cDNA using the RT² First Strand Kit, mixed with RT² qPCR Master Mix, containing SYBR Green, according to manufacturer's instructions (Qiagen, Hilden, Germany). In this custom array, the following nonregulated genes (genes of interest) were used for normalization of the fold change expression data calculations: Glyceraldehyde-3-phosphate dehydrogenase, β 2-microglobulin.

Chemical Reagents

PE, ACh, and SNP were obtained from Sigma-Aldrich (Taufkirchen, Germany). Custodiol and Tiprotec were obtained from Köhler Chemie GmbH.

Statistical Analysis

Statistical analysis was performed using SPSS Statistics 24 (IBM Corp, Armonk, NY). On the basis of our previous investigations,^{12,16} we calculated the Cohen d (in that case endothelium-dependent vasorelaxation: mean 1 = 90, mean 2 = 25, SD 1 = 5, SD 2 = 16, Cohen d = 5). We calculated with Cohen d = 2.2 in this study. Thus, in our present study, we needed 6 pigs per group. To improve the effect size, we used 8 pigs per group in our study. Data were tested for normal distribution with the Shapiro–Wilk test. In the case of vasorelaxation studies on ITA rings a repeated measurement analysis of variance was carried out with “vasorelaxant dose” as within-subject factor (repeated measures) and “graft storage type” as between-subject factor (independent groups). *P* values for dose, group, and Dose $\times$ Group interaction were calculated. Bonferroni post hoc test for multiple comparisons were performed. Other results were compared using 1-way analysis of variance after pairwise *t* test including Bonferroni correction ($R_{\max}$ to ACh, CD-31, nitrotyrosine, ICAM-1, TUNEL, eNOS) or Kruskal–Wallis test after pairwise *U* test including Bonferroni correction (VCAM-1, caspase-3). Results are presented as mean $\pm$ SD. A value of *P* < .05 was considered indicative of significance after Bonferroni correction.

RESULTS

Vasomotor Function

After reperfusion, a significant impairment of endothelium-dependent vasorelaxation to ACh of ITA rings was shown in the free ITA groups compared with the control (*P* < .05). In contrast, the in situ ITA had equivalent endothelium-dependent vasorelaxation compared with the control (Figure 2). Tiprotec improved the endothelium-dependent function of ITA rings compared with the saline group. Endothelial dysfunction was indicated by reduced maximal relaxation of ITA rings to ACh and a rightward shift of the concentration response curve compared with

FIGURE 2. Vasomotor function of internal thoracic artery (ITA) grafts. Acetylcholine-induced endothelium-dependent vasorelaxation is shown after 2 hours of reperfusion. Values represent mean $\pm$ SD. In the case of vasorelaxation studies on ITA rings a repeated measurement analysis of variance was carried out with “vasorelaxant dose” as within-subject factor (repeated measures) and “graft storage type” as between-subject factor (independent groups). Bonferroni post hoc tests for multiple comparisons were performed. Dose-effect *P* value < .0001; group effect *P* value < .0001; dose + group effect *P* value = .0057. Values of maximal relaxation were compared using 1-way analysis of variance after pairwise *t* test including Bonferroni correction. *N*, Number of animals; *n*, number of rings; *IE*, logarithm base. **P* < .05 versus control ITA. #*P* < .05 versus in situ ITA.

the control. There was no significant difference in $R_{\max}$ for endothelium-independent vasorelaxation of the ITA rings to SNP among the experimental groups. Contractile responses of ITA rings to PE (10^{-6} M) are shown in Table 2.

Oxidative Stress and Apoptosis

An increased density of TUNEL-positive nuclei indicates severe DNA fragmentation in the arterial wall (intima and media) in the experimental groups. Significantly increased DNA strand breaks (Figures 3 and 4) were observed throughout ITA groups, however TUNEL-positive cells in the in situ ITA and free ITA:Tiprotec group were significantly lower compared with the free ITA:saline and Custodiol groups.

Immunostaining for nitrotyrosine showed significantly enhanced immunoreactivity in the wall of free ITA grafts. The control and in situ ITA group showed decreased nitrotyrosine immunoreactivity. Preservation of the ITA in Tiprotec significantly reduced oxidative stress compared with the free ITA:saline group (Figures 3 and 4).

An increased density of caspase-3-positive cells indicated a severe apoptosis rate in the free ITA:saline and Custodiol groups. No significant changes were found in the vessel wall for caspase-3 staining in the in situ and free ITA:Tiprotec groups compared with the control (Figures 3 and 4).

eNOS Expression

Immunostaining for eNOS showed significantly enhanced immunoreactivity in the wall of ITA rings of the control and in situ ITA groups. The eNOS score was significantly lower in the free ITA groups (Figure 4).

Expression of Adhesion Molecules (CD-31, ICAM-1, VCAM-1)

The inner walls of all ITA segments were covered with endothelium showing a positive CD-31 reaction. In the control group the endothelial surface showing a positive reaction for the CD-31 antigen (Figure 3) was estimated as $87 \pm 2\%$, whereas in most free ITA vessels this rate was significantly lower ($63 \pm 6\%$ in the saline group, $70 \pm 8\%$ in the Custodiol group; $P < .05$). The CD-31 reaction was not reduced in the in situ ITA and free ITA: Tiptotec group compared with the control.

Immunohistochemical staining revealed high expression of VCAM-1 and ICAM-1 in the free ITA groups. Control ITA grafts barely expressed ICAM-1 and VCAM-1. Immunohistochemical scores from the semiquantitative evaluation were significantly lower in the in situ ITA group compared with the free ITA groups (Figure 4).

Changes in Gene Expression

We determined the expression patterns of 38 genes. The up- and downregulated gene expressions are shown in Figure 5.

DISCUSSION

In our experimental study, off-pump CABG was used to simulate the clinical conditions witnessed during arterial revascularisation. This allowed both the observation of reperfusion injury's early effect on the endothelium of an ITA graft, and the evaluation of the preservation solution's effect on the vascular function of the ITA graft.

Pathomechanisms of Graft Injury

In the current study, we showed, to our knowledge, for the first time, that the vasomotor function of a stored, free ITA graft preserved in routinely used preservation solutions (saline/Custodiol) was severely damaged during the early stage of reperfusion injury. The results of the present experiment are in good agreement with previous studies¹⁷ and our previous reports on endothelial dysfunction of arterial grafts, in which severe endothelial damage also occurred immediately after the initiation of reperfusion.^{12,16,18} Furthermore, the reported functional endothelial impairment might also correlate to clinical findings after CABG, when the early postoperative complications (vasospasm/occlusion of bypass graft) often occurred during the first 24 hours.¹⁹ An interesting new finding of our study is that the vasomotor function of the in situ ITA graft was similar to the control ITA group.

Ischemia followed by rapid reintroduction of molecular oxygen per se results in a burst of ROS during the first minutes of reperfusion.²⁰ Activated leukocytes also generate a number of ROS. The luminal surface of blood vessels is the most exposed tissue to the deleterious effects of ROS, which lead to endothelial cell death. There is now good evidence supporting an elevated production of ROS' contribution to cell dysfunction/death via induction of oxidative damage to cell macromolecules.²⁰ Peroxynitrite (highly reactive coupling product of NO and superoxide) was also shown to be an important mediator of tissue injury in ischemia-reperfusion (IR) injury.²¹ Our immunohistological study revealed marked nitrotyrosine staining in the free ITA groups, however, the in situ ITA graft was free from injury. Furthermore, the results of TUNEL and caspase-3 staining showed marked damage of the endothelial structure in the free ITA groups as a consequence of increased oxidative stress and apoptosis in the endothelium. We also detected extensive endothelial cell death, measured by a lower CD-31 expression in the

TABLE 2. Values of $R_{\max}$ (% and g) and pD_2 to ACh, to SNP, and contraction forces induced by PE (10^{-6} M) in ITA rings in control, in situ ITA, free ITA:saline, free ITA:Custodiol, and free ITA:Tiptotec groups

	Control (N = 6; n = 20)	In situ ITA (N = 8; n = 20)	P in situ versus control	Free ITA:saline (N = 8; n = 20)	P saline versus control	P saline versus in situ
$R_{\max}$ to ACh, %	87 ± 17	73 ± 28	1.000	$25 \pm 16^{*,\dagger}$	<.0001	<.0001
pD_2 to ACh	7.2 ± 0.5	7.1 ± 0.4	1.000	$6.7 \pm 0.6^*$	.036	.001
$R_{\max}$ to SNP, %	82 ± 23	84 ± 22	1.000	93 ± 10	1.000	1.000
pD_2 to SNP	4.9 ± 1.1	4.6 ± 1.5	1.000	5.0 ± 1.0	1.000	1.000
PE, % of KCl	56.9 ± 13	$81.0 \pm 22^*$	.024	$102.6 \pm 20^*$	<.0001	.064
Maximal contraction to KCl, g	7.1 ± 1.9	$4.1 \pm 1.8^*$	<.0001	$3.5 \pm 1.7^*$	<.0001	.295
Maximal contraction to PE, g	4.0 ± 0.9	3.1 ± 1.0	1.000	3.5 ± 1.7	1.000	1.000
Blood flow after 2-hour reperfusion, mL/min		29 ± 16	1.000	30 ± 14	1.000	1.000

Values are presented as mean $\pm$ SD except where otherwise noted. Significant P values are adjusted using Bonferroni correction for multiple tests. n = Number of rings; ITA, internal thoracic artery; $R_{\max}$, maximal vasorelaxation; ACh, acetylcholine; pD_2 , negative value for log half-maximal response; SNP, sodium nitroprusside; PE, phenylephrine; N, number of animals. * $P < .05$ versus control. $\dagger P < .05$ versus in situ graft. $\ddagger P < .05$ versus Custodiol. $\S P < .05$ versus saline.

free ITA graft groups. Thus, endothelial integrity and function were also severely damaged in the free ITA groups. Interestingly, the vasomotor function and the integrity of the endothelial layer of the in situ ITA graft was free from damage.

Endothelial dysfunction is also defined as the decreased synthesis, release, and/or activity of endothelium-derived NO. Endothelial cell activation by proinflammatory cytokines could lead to endothelial dysfunction by inhibiting eNOS expression and decreasing NO bioavailability through the induction of ROS. It has also been shown that NO donors can prevent intimal hyperplasia in animal models, which is the main cause of restenosis.²² On the basis of these observations, we determined the eNOS content using immunohistochemistry, as well as by measuring eNOS expression. As shown in Figure 4, immunoreactivity of eNOS was significantly higher in the in situ ITA group compared with those of the free ITA groups, thus it might theoretically be an explanation of the impaired vasorelaxation of the stored, free ITA grafts. In accordance with our results, much of the data derived from studies of IR-induced vascular dysfunction are consistent with the role of NO in the changed endothelium-dependent responses.²³

During the early phase of reperfusion, several adhesion molecules are upregulated. On a functional level, adhesion molecules (VCAM-1, ICAM-1) lead to the recruitment of leukocytes to the endothelium and their migration into the tissue. In the current study we reported an overexpression of ICAM-1 and VCAM-1 in the free ITA groups. This finding is consistent with previous results in different models of regional IR.²⁴ Several studies showed an increased concentration of ICAM-1/VCAM-1 after leukocyte-mediated heart injury.²⁵ The importance of ICAM-1 and VCAM-1 in neutrophil-mediated injury was also shown.²⁶ This agrees with our findings; a significant upregulation of ICAM-1 and VCAM-1 was shown in the

free ITA graft groups, however the expression remained low in the in situ ITA group. In accordance with immunohistological results, we also detected a higher mRNA expression of ICAM-1 and VCAM-1 in the free ITA groups.

Furthermore, the mRNA expression of hypoxia-inducible factor 1 (HIF-1), vascular permeability factor (VEGF), and matrix metalloproteinase-9 (MMP-9) were upregulated after reperfusion. Bateman and colleagues²⁷ reported that during hypoxia, HIF-1, on an mRNA level, was significantly elevated very rapidly in the first 2 hours, which was followed by the increased levels of target genes (for example VEGF). There is evidence that endothelial injury is a primary cause of NIH, which is the basic pathology of vascular disease.²⁸ VEGF and MMP-9 have an important role directly in the progress of NIH.^{29,30} Guo et al reported that MMP-9 expression is increased during the first 2 weeks after endothelial injury, and thus a higher expression level of MMP-9 later contributes to intimal hyperplasia.³⁰ HIF-1 has also been reported to be involved in NIH after vascular injury.³¹

Effect of Different Preservation Solutions on Endothelial Function

The available methods of preservation of free vascular grafts have a deleterious effect on the endothelium. Experimental studies reported poor preservation capacity of Custodiol and saline solution.^{12,16,18} In accordance with the literature, we also showed that Custodiol and saline are not optimal preservation solutions for the storage of free ITA grafts, as we showed by a severely impaired vasomotor function and changed structure of the stored, free ITA graft.

It has previously been reported that ROS play an important role in NIH, which leads to restenosis.¹³ Antioxidant drugs that reduce ROS levels have been described as important therapeutic targets for the treatment of restenosis.^{13,32}

TABLE 2. Continued

Free ITA:Custodiol (N = 8; n = 20)	Custodiol versus control	Custodiol versus in situ	Custodiol versus saline	Free ITA:Tiprotec (N = 8; n = 20)	Tiprotec versus control	Tiprotec versus in situ	Tiprotec versus saline	Tiprotec versus custodiol
32 ± 24*,†	<0.0001	0.001	1.000	45 ± 18*,†	0.002	0.036	0.328	1.000
6.7 ± 0.6*,†	0.009	<0.0001	1.000	7.3 ± 0.4‡,§	1.000	1.000	0.001	0.0001
81 ± 22	1.000	1.000	1.000	84 ± 16	1.000	1.000	1.000	1.000
4.4 ± 0.8*	0.004	1.000	0.083	4.8 ± 0.9	1.000	1.000	1.000	0.838
109.2 ± 39*,†	<0.0001	0.019	0.814	66.8 ± 19‡,§	0.170	0.601	<0.0001	<0.0001
3.4 ± 1.0*	<0.0001	0.093	0.555	5.5 ± 1.7*,†,§	<0.0001	0.147	0.003	<0.0001
3.6 ± 0.9	1.000	1.000	1.000	3.6 ± .0.9	1.000	1.000	1.000	1.000
29 ± 11	1.000	1.000	1.000	27 ± 7	1.000	1.000	1.000	1.000

FIGURE 3. Representative photomicrographs of immunohistochemical stainings for terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (*TUNEL*), CD-31, nitrotyrosine, caspase-3, eNOS, VCAM-1, and ICAM-1. Groups are indicated by (A) for control, (B) for in situ graft, (C) for free graft:saline, (D) for free graft:Custodiol, and (E) for free graft:Tiprotec. *eNOS*, Endothelial nitric oxide synthase; *VCAM-1*, vascular cell adhesion protein 1; *ICAM-1*, intercellular adhesion molecule-1.

On the basis of novel insights in cell/tissue injury during IR, a new preservation solution (Tiprotec) was developed. Tiprotec was modified in 4 important points to enhance

the protective capacity of Custodiol solution: (1) addition of iron chelators to reduce iron-dependent injury, (2) partial substitution of histidine by N- α -acetyl-L-histidine to inhibit

FIGURE 4. Semiquantitative scoring of immunohistochemical stainings for terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (*TUNEL*), nitrotyrosine, caspase-3, vascular cell adhesion protein 1 (*VCAM-1*), and intercellular adhesion molecule-1 (*ICAM-1*); N = 6 animals, n = 12 for control, and N = 8 animals, n = 16 samples for other groups; CD-31 (N = 6 animals, n = 12 samples), and eNOS (N = 6 animals, n = 18 for control and N = 8 animals, n = 24 samples for other groups). Individual points in each group overlay box plot. The box defines the 25th to 75th interquartile range and median (line) and mean (empty square) values are shown within the box. Results were compared using 1-way analysis of variance after pairwise *t* test including Bonferroni correction (CD-31, nitrotyrosine, ICAM-1, *TUNEL*, eNOS) and Kruskal-Wallis test after pairwise *U* test including Bonferroni correction (VCAM-1, caspase-3). Global statistical results: caspase-3 $P = .002$; CD-31 $P = .001$, eNOS $P < .0001$, ICAM-1 $P < .0001$, VCAM-1 $P < .0001$, nitrotyrosine $P < .0001$, *TUNEL* $P = .0004$. * $P < .05$ versus control ITA; # $P < .05$ versus in situ ITA; § $P < .05$ versus free ITA:Custodiol; \$ $P < .05$ versus free ITA:Tiprotec. *eNOS*, Endothelial nitric oxide synthase.

FIGURE 5. Up- and downregulated genes. A, Control ITA versus in situ ITA (scatter plot). B, Control ITA versus free ITA:saline (scatter plot). C, Control ITA versus free ITA:Custodiol (scatter plot). D, Control ITA versus free ITA:Tiprotec (scatter plot). Fold change ($2^{-\Delta\Delta Ct}$) is the normalized gene expression ($2^{-\Delta\Delta Ct}$) in the test sample divided by the normalized gene expression ($2^{-\Delta\Delta Ct}$) in the control sample. The *black line* indicates fold changes ($2^{-\Delta\Delta Ct}$) of 1. The *pink lines* indicate the desired fold change in gene expression threshold, defined by 2. The *red symbols* identify upregulated genes, and the *blue symbols* downregulated genes. RNA from $n = 5$ pigs per group was isolated and the expression analyzed using polymerase chain reaction array from SA Biosciences (Qiagen, Hilden, Germany). *SERPINE1*, Plasminogen activator inhibitor-1; *HIF1A*, hypoxia-inducible factor 1-alpha; *IL6*, interleukin-6; *ICAM-1*, intercellular adhesion molecule-1; *VCAM-1*, vascular cell adhesion protein 1; *IL1B1*, interleukin-1 beta; *PRKCA*, protein kinase C alpha; *IL8*, interleukin-8; *VEGFA*, vascular permeability factor; *TNF*, tumor necrosis factor alpha; *VWF*, Von Willebrand factor; *GRIN2D*, glutamate ionotropic receptor NMDA type subunit 2D; *IL10*, interleukin-10; *CIDE-B*, cell-death-inducing DNA-fragmentation-factor-like effector B; *MMP-9*, matrix metalloproteinase 9; *IL7*, interleukin-7; *C-JUN*, proto-oncogen c-jun; *BCL2*, B-cell lymphoma 2; *CAMK1*, calcium-calmodulin-dependent protein kinase type 1; *CASP3*, caspase-3; *SOD1*, superoxide dismutase; *BAX*, Bcl-2-like protein 4; *NOS3*, endothelial nitric oxide synthase.

the histidine-induced cytotoxicity, (3) addition of cardio-protective amino acids, and (4) increased potassium concentration.¹²

In accordance with the previous results, we also showed a superior preservation capacity of Tiprotec over Custodiol

and saline solutions. Furthermore, among the free ITA groups, the functional and structural impairment of the ITA graft was partly ameliorated by Tiprotec. An increased CD-31, eNOS staining, and reduced TUNEL-positive nuclei, ICAM-1 and VCAM-1 score, and nitro-oxidative

stress were also detected in the Tiprotec group compared with the saline and Custodiol groups. Our findings also correlate to in vitro and in vivo studies, in which the detrimental effect of saline and Custodiol on the endothelium of grafts was reported.^{12,33}

CONCLUSIONS

To the best of our knowledge, this is the first study that describes a high vulnerability of the ITA graft to reperfusion injury: the endothelial function and integrity of stored, free ITA grafts was largely abolished after reperfusion in daily used preservation solutions (saline, Custodiol). Among the free ITA groups, the vascular impairment of the ITA graft was partly ameliorated by the new preservation solution, Tiprotec. However, the endothelial cells of the in situ ITA graft showed no signs of IR injury. In conclusion, avoidance of daily used preservation solutions (saline/Custodiol) and preparation of the free arterial grafts similar to the in situ grafts should be considered.

Limitations

The off-pump model in this study was selected as a suitable model to evaluate in vivo IR injury. However, this model has certain limitations. Porcine ITA tissue differs from arterial grafts for CABG in the structure of the vessel wall, which limits the transferability of the results. Furthermore, direct correlation between graft patency and early vascular impairment was not investigated in our study. Even if upregulated MMP-9 and VEGF are indicative of NIH at later time points, we have no evidence at this point, so further studies are warranted. Furthermore, we have yet not tested free ITA grafts, which are prepared similarly to the in situ grafts, which are not subjected to storage.

Conflict of Interest Statement

Dr Szabó has a research grant from the Köhler Chemie GmbH (Bensheim, Germany) for another product (Custodiol). All other authors have nothing to disclose with regard to commercial support.

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