

The impact of distension pressure on acute endothelial cell loss and neointimal proliferation in saphenous vein grafts

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Abstract

OBJECTIVES: We aimed to determine the extent of acute endothelial cell loss and neointimal proliferation in the long-term in saphenous vein grafts (SVGs) exposed to defined distension pressures.

METHODS: During routine competence testing of SVGs for coronary artery bypass grafting (CABG), blinded peak pressure measurements were performed in 10 patients. In an experimental set-up, distension pressure-related endothelial damage was studied in the SVGs of 20 patients. In a subgroup ($n = 10$), each patient's SVG was divided into segments and subjected to four constant pressures (50, 100, 150 and 300 mmHg) for 30 min each. In another subgroup ($n = 10$), SVGs were exposed to a short phase of high pressure (low pressure followed by 300 mmHg for 5 min). Acute endothelial cell loss was quantified by CD31-immunostaining. After 2 weeks of organ culture, the neointimal proliferation was evaluated using histomorphometry. Pressure-related damage was compared with damage at baseline (0 mmHg).

RESULTS: During routine competence testing for CABG, we revealed a median peak pressure of 355 mmHg (range: 240–639 mmHg). In the experimental set-up, significant acute endothelial cell loss occurred at all tested distension pressures: at 50 mmHg, the median endothelial cell loss was 29% (range: 20–51%, $P = 0.015$), at 100 mmHg 54% (range: 37–69%, $P < 0.001$), at 150 mmHg 75% (range: 41–88%, $P < 0.001$), at 300 mmHg 91% (range: 63–100%, $P < 0.001$) and at short high-pressure exposure 65% (range: 49–82%, $P < 0.001$) in comparison with 20% (range: 0–44%) at baseline. Significant neointimal proliferation occurred when a distension pressure of 50 mmHg was exceeded: at 50 mmHg, median neointimal proliferation was 97 μm (range: 60–380 μm , $P = 0.176$), at 100 mmHg 168 μm (range: 100–600 μm , $P = 0.001$), at 150 mmHg 183 μm (range: 160–440 μm , $P < 0.001$) at 300 mmHg 347 μm (range: 190–590 μm , $P < 0.001$) and at short high-pressure exposure 130 μm (range: 60–410 μm , $P = 0.02$) in comparison with 90 μm (range: 60–170 μm) at baseline.

CONCLUSIONS: *In vitro* exposure of SVGs to low distension pressure ranges causes significant acute endothelial cell loss and crucial long-term damage, namely neointimal proliferation.

Keywords: Distension pressure • Endothelial cell loss • Neointimal proliferation • Saphenous vein grafts

INTRODUCTION

Saphenous vein grafts (SVGs) are routinely used in coronary artery bypass grafting (CABG). However, the patency rates of SVGs post-CABG are unsatisfactory [1]. After 10 years, ~65% of SVGs are patent and 50% of those show angiographic signs of atherosclerosis. Twenty years after CABG, only 25% of SVGs provide sufficient blood flow [2–4]. The development of neointimal proliferation correlates directly with poor SVG patency rates [5].

Uncontrolled hydrostatic distension is common during routine competence testing of SVGs in CABG. It causes considerable damage not only to the endothelium, but also to the media and adventitia [6]. Furthermore, the surgical manipulation during SVG harvesting itself causes disruption of endothelial cell viability [7].

This surgically induced endothelial cell damage may result in early SVG thrombosis. In the long-term, neointimal proliferation, also called the 'vein graft disease,' can develop with atherosclerosis progression. Neointimal proliferation follows a pathogenic sequence with an initial proliferation of medial smooth muscle cells in response to a number of growth factors and cytokines released from platelets, endothelial cells and macrophages [7]. Activated smooth muscle cells migrate from the medial to the intimal layer of venous vascular walls, and transform from a contractile to a proliferative phenotype. Synthesis and deposition of extracellular matrix by activated smooth muscle cells lead to a progressive increase in intimal fibrosis and a reduction in cellularity. A neointima develops, forming the matrix for graft atherosclerosis. The consequence is a non-patent SVG [8, 9].

Here, in a first step, we performed pressure monitoring during a routine competence testing of SVGs to assess peak distension pressures. Then, in an experimental set-up, we determined the extent of acute endothelial cell loss and, more importantly, long-term neointimal proliferation in SVGs in response to defined distension pressures.

MATERIALS AND METHODS

Approval was obtained from the local institutional review board for the investigation of human vein grafts. All participants granted informed consent. This study was conducted in accordance with the Helsinki Declaration.

Pressure monitoring during SVG competence testing

Pressure monitoring was performed with a pressure transducer (DTX Plus™, BD, Franklin Lakes, NJ, USA) to assess peak distension pressures during routine competence testing of SVGs in 10 patients undergoing CABG. For competence testing, a 20-ml syringe filled with physiological saline solution at room temperature (21°C) was used by an experienced surgeon. The surgeon was not aware of the measured peak pressure (blinded measurement).

Vein graft harvesting and processing for the experimental set-up

SVGs of a total of 20 patients undergoing routine CABG were harvested with an atraumatic technique as described by Souza [10, 11]. The distal parts of the non-distended greater saphenous veins were utilized for study samples. The proximal parts were used as bypass grafts for CABG. The SVGs were immediately transported in physiological saline solution (21°C) from the operating room to the laboratory. The time elapsed from SVG harvesting to laboratory processing did not exceed 60 min.

Exposure to distension pressures

In a subgroup of 10 patients, each patient's SVG was divided into segments and exposed to four different constant distension pressures (50, 100, 150 and 300 mmHg) for 30 min each. In another subgroup of 10 patients, the SVGs were exposed to a short phase of high pressure (50 mmHg for 25 min followed by 300 mmHg for 5 min). For this purpose, SVG segments were cannulated, filled with physiological saline solution (21°C) and exposed to the distinct distension pressure using a pressure calibrator (Heraeus Holding, Hanau, Germany). After pressure exposure, all the squeezed parts (cannulation site and clip area) of the SVG segments were removed and the remaining part was divided into two lengths. One length was used to determine the acute endothelial cell loss by means of CD31-immunostaining. The other length was transferred to an organ culture in order to study the neointimal proliferation by histomorphometry. Pressure-related endothelial cell loss and neointimal proliferation

in each subgroup were compared with a pooled baseline (0 mmHg) of all 20 patients.

CD31-immunostaining

CD31-immunostaining was performed to evaluate endothelial cell loss, as CD31 is a surface marker of endothelial cells. SVG segments were fixed in 4% paraformaldehyde for 18 h, dehydrated and paraffin-embedded. Three cross-sectional slices from different parts of each SVG segment were analysed in order to obtain representative results. Slices were deparaffinized. Antigen retrieval was performed for 30 min at 98°C in an antigen retrieval solution (=10 mM TRIZMA Base, 1 mM Ethylenediaminetetraacetic acid solution, 0.05% Tween 20, pH 9.0). The CD31 antibody (Dako, Glostrup, Denmark; 1:30 diluted in 0.05 mol/l Tris-HCl buffer with 1% albumin) was added for 60 min. Blocking, staining and antibody detection were performed according to the kit protocol (Dako), whereas counterstaining was performed with haematoxylin (Merck, Whitehouse Station, NJ, USA). Digital images of each slide were recorded in high resolution and CD31⁺ endothelial cells on the intimal surface were identified and quantified (ImageJ 1.32 for Macintosh, National Institutes of Health, USA).

The ratio of CD31⁺ endothelial cell surface to the total intimal surface of the cross-section (CD31⁺ endothelial cell surface and de-endothelialized tunica intima) demonstrated the percentage of endothelial cell coverage. The reciprocal value gave the percentage of endothelial cell loss. Endothelial cell loss results for a distinct distension pressure are expressed as the median value of three measurements in different parts of the SVG segment. Evaluation of immunohistochemistry was performed by an experienced pathologist in a blinded setting.

Organ culture

Organ culture of SVGs as a model of neointimal proliferation was described by Porter *et al.* [12] and validated by Castronuovo [13]. SVG segments were sliced longitudinally and pinned on a mesh placed on a silicon pad (with the endothelial side facing up). This was transferred to a Petri dish containing 10 ml RPMI 1640 culture medium with 30% foetal bovine serum, 4 units/ml heparin, 25 g/ml gentamycin, 2.5 g/ml amphotericin, 100 units/ml penicillin, 100 g/ml streptomycin and L-glutamine (Sigma-Aldrich, St. Louis, MO, USA). The culturing conditions in the incubator (Hera Cell, Hanau, Germany) were 37°C and 5% carbon dioxide. Culture medium was renewed every 48 h.

Histomorphometric quantification of cross-sectional neointimal proliferation

After 14 days of organ culture, SVG segments were paraffin-embedded. Again, from each SVG segment, three cross-sectional slices from different parts were analysed in order to obtain representative results. Slices were deparaffinized and stained with Elastica van Gieson (Acustain Elastic Stain Kit, HT25A, Sigma-Aldrich) to visualize the internal elastic membrane. This membrane discriminates the tunica intima from the media of blood vessel walls. Images of each slide were recorded

digitally and quantitative histomorphometric analysis of the neointimal thickness was performed (ImageJ 1.32 for Macintosh, National Institutes of Health, Bethesda, MD, USA). On each slice, neointimal proliferation was measured at 30 places across the high-power field and median values were calculated. Again, neointimal proliferation results for a distinct distension pressure are expressed as the median value of three measurements from different parts of the SVG segment. Evaluation of histomorphometry was performed by an experienced pathologist in a blinded setting.

Patient demographics

Preoperative cardiovascular risk factors were determined in the 20 patients enrolled in the experimental study. Eighteen (90%) patients had hypertension, 6 (30%) diabetes mellitus and 13 (65%), a history of smoking. The mean \pm SD preoperative total cholesterol level was 162.4 ± 26.8 mg/dl and the mean triglyceride level was 145.5 ± 46.6 mg/dl. Pre-operative medication consisted of aspirin in 19 (95%), statin in 15 (75%), betablocker in 17 (85%), angiotensin-converting-enzyme inhibitor in 11 (55%) and nitroglycerine in 12 (60%) patients. Arteriosclerosis was the underlying pathology in all patients.

Data analysis

Due to a non-parametric data distribution, results on endothelial cell loss and neointimal proliferation are presented in median and range (minimum–maximum). The Mann-Whitney *U*-test was used to compare pressure-related endothelial damage with damage in a pooled baseline (0 mmHg). *P*-values < 0.05 were considered statistically significant. For statistical analysis, SPSS 12.0 (Chicago, IL, USA) was used.

RESULTS

Blinded peak pressure monitoring during competence testing of SVGs in CABG

A median peak pressure of 355 mmHg (range: 240–639 mmHg) was revealed during routine competence testing of SVGs by experienced surgeons ($n = 10$).

Endothelial cell loss in SVGs exposed to defined distension pressures

Significant endothelial cell loss occurred at all tested distension pressures. The extent of acute endothelial cell loss increased with increasing pressure magnitude: at 50 mmHg the median endothelial cell loss was 29% (range: 20–51%, $P = 0.015$), at 100 mmHg 54% (range: 37–69%, $P = 0.001$), at 150 mmHg 75% (range: 41–88%, $P < 0.001$), at 300 mmHg 91% (range: 63–100%, $P < 0.001$) and at short high-pressure exposure 65% (range: 49–82%, $P < 0.001$) in comparison with 20% (range: 0–44%) at baseline (0 mmHg); (Figs. 1A and 2).

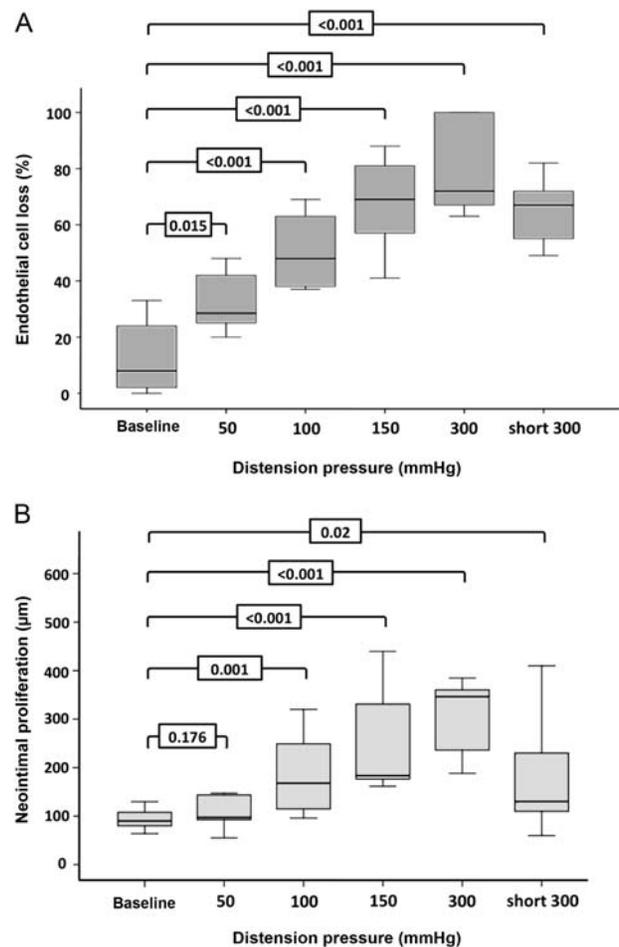


Figure 1: Boxplot diagrams showing (A) the extent of acute endothelial cell loss in response to defined constant distension pressures (50, 100, 150 and 300 mmHg) for 30 min each and to a short phase of high pressure (50 mmHg for 25 min followed by 300 mmHg for 5 min) in comparison with baseline (0 mmHg), (B) the extent of neointimal proliferation after 2 weeks of organ culture in response to defined constant distension pressures (50, 100, 150 and 300 mmHg) for 30 min each and to a short phase of high pressure (50 mmHg for 25 min followed by 300 mmHg for 5 min) in comparison with baseline (0 mmHg). The line within the boxes indicates the median. The top edge of the boxes represents the 75th percentile; the bottom edge, the 25th percentile. The range is shown as a vertical line. Whiskers demarcate the minimum and maximum, excluding outliers.

Neointimal proliferation in vein grafts exposed to defined distension pressures

After 2 weeks of organ culture, significant neointimal proliferation occurred in SVGs when a distension pressure of 50 mmHg was exceeded: at 50 mmHg, median neointimal proliferation was 97 μ m (range: 60–380 μ m, $P = 0.176$), at 100 mmHg 168 μ m (range: 100–600 μ m, $P = 0.001$), at 150 mmHg 183 μ m (range: 160–440 μ m, $P < 0.001$) at 300 mmHg 347 μ m (range: 190–590 μ m, $P < 0.001$) and at short high-pressure exposure 130 μ m (range: 60–410 μ m, $P = 0.02$) in comparison with 90 μ m (range: 60–130 μ m) at baseline (0 mmHg); (Figs. 1B and 3).

DISCUSSION

The present study analysed the extent of endothelial damage in SVGs in response to defined distension pressures. In addition to

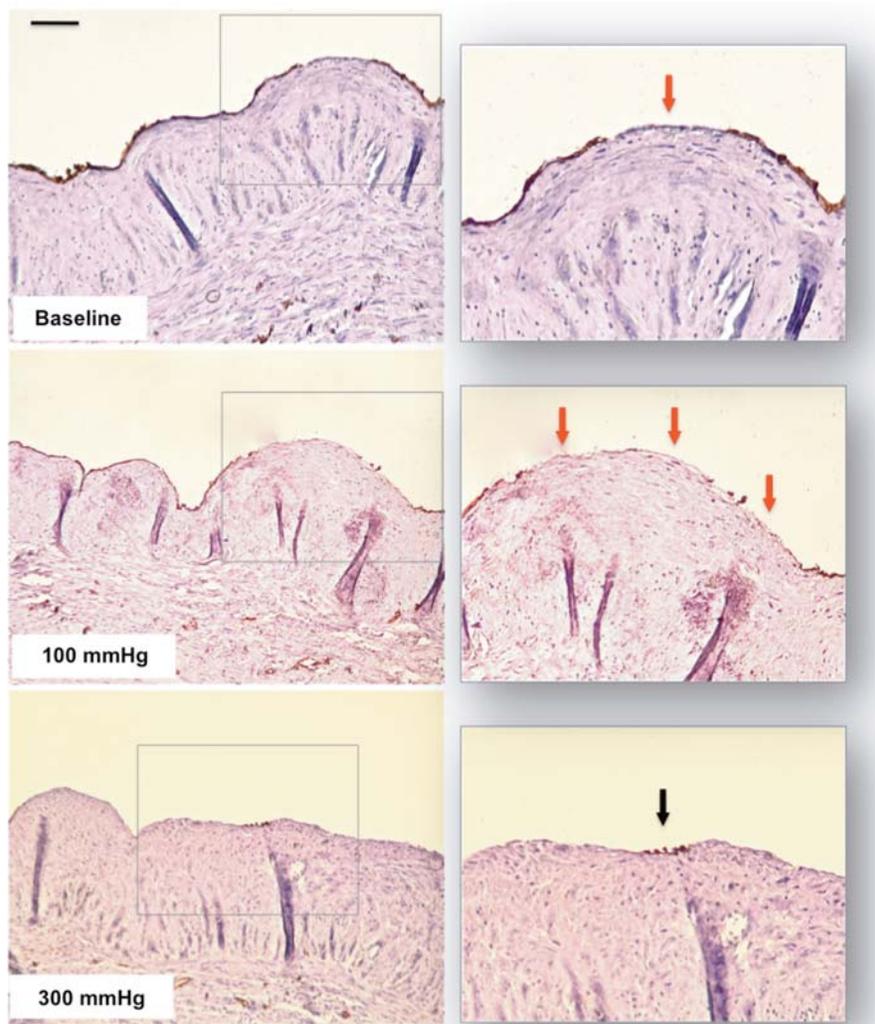


Figure 2: Acute endothelial cell loss in SVGs exposed to defined distension pressures. Representative examples for baseline (0 mmHg), 100 and 300 mmHg are shown. CD31⁺ intimal endothelial cell alignment is stained in brown. Upper row: At baseline the vein graft shows a widely intact intimal surface. The red arrow in the magnification marks a small de-endothelialized area. Middle row: At a 100 mmHg distension pressure, distinct endothelial cell loss is marked with three red arrows. Lower row: A distension pressure of 300 mmHg causes an almost complete endothelial cell loss and flattened intimal surface. The black arrow marks an isolated endothelial cell island. Bar indicates 100 μ m.

acute damage, we report here on the long-term effect of pressure exposure in SVGs, namely neointimal proliferation.

We and others found that peak distension pressures of several hundred mmHg routinely occur during competence testing in SVGs [14]. In the present study, median peak pressures of \sim 350 mmHg were reached, while Roubos *et al.* [14] describe even higher peak pressures of \sim 480 mmHg. Together, these findings underline the necessity for an awareness of pressure-related impairment of SVG quality and the need for data on the extent of pressure-related damage to the endothelium.

It is remarkable that a significant endothelial cell loss of 29% already occurred at a distension pressure as low as 50 mmHg. Of note, at a distension pressure of only 100 mmHg, the loss of viable endothelial cells was already 54% in comparison with 20% at baseline. This means that endothelial cell loss increased by more than 2.5 times. The literature only rarely contains data on the extent of endothelial damage in response to pressure exposure. Weiss *et al.* [15] investigated endothelial cell loss in SVGs exposed to defined pressures between 50 and 1000 mmHg for a 5 min 'manipulation' phase (with intermittent pressure

reductions to imitate the competence testing procedure) followed by a 45 min storage phase, during which the initially defined pressure fell slowly and continuously. After exposure to low pressures, they detected a high degree of de-endothelialized intimal surface, which is in keeping with our findings. They report an endothelial cell loss of 39–49% (depending on the type of planimetry used for evaluation) at a distension pressure of only 50 mmHg and a loss of 47–54% at 100 mmHg. In addition, our findings on endothelial cell loss after brief exposure to 300 mmHg match the results of Weiss *et al.* The 65% endothelial cell loss observed in our study lies well between the 56–58% at 200 mmHg and the 85–92% cell loss at 500 mmHg found in their study. Roubos *et al.* [14] also report a high degree of endothelial cell loss, namely 56%, at a mean pressure of 136 mmHg (calculated from the area under the pressure curve) during harvesting and routine competence testing of SVGs. Their data again correspond with our rate of 54% at a constant exposure to 100 mmHg over 30 min. One further study assessed structural effects (in addition to biochemical and functional effects) of pressure exposure in SVGs, although not quantitatively [16].

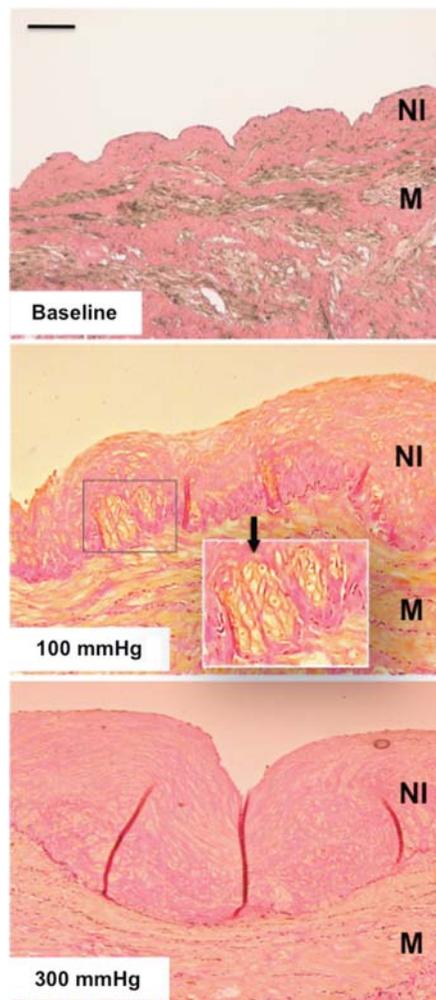


Figure 3: Neointimal proliferation in cultured SVGs exposed to defined distension pressures. Representative examples for baseline (0 mmHg), 100 and 300 mmHg are shown. Slides are stained with Elastica van Gieson. Upper row: At baseline, a delicate neointima (NI) and a tunica media (M) with typical smooth muscle cell alignment are present. Middle row: At a 100 mmHg distension pressure, distinctive neointimal proliferation developed. Smooth muscle cells migrate from the medial to the intimal layer and transform from a contractile to a proliferative phenotype (black arrow). Lower row: At a 300 mmHg distension pressure, massive neointimal proliferation developed. Neointimal proliferative smooth muscle cells secrete matrix substances causing massive fibrosis with decreased neointimal cell content. Bar indicates 100 μ m.

Most importantly, the present study simulated the long-term effects of increasing distension pressures in SVGs and studied these in an organ culture model. Of note, significant neointimal proliferation was observed in response to a distension pressure as low as 100 mmHg, and this pressure almost doubled neointimal thickness when compared with baseline. Neointimal proliferation further increased with increasing distension pressures, reaching almost 400% of baseline neointimal thickness at 300 mmHg. The long-term effect of pressure exposure, namely neointimal proliferation, is probably the crucial endpoint for estimating the *in vivo* relevance of pressure-related SVG damage. To the best of our knowledge, we provide here the first data on neointimal proliferation in response to defined pressure exposure in SVGs.

We did not investigate whether there is a causal link between pressure-related endothelial cell loss and neointimal proliferation

in our experimental model. *In vivo*, endothelial cells that are activated in response to damage as well as adherent platelets and macrophages are known to release a number of growth factors and cytokines. These factors can trigger proliferation of medial smooth muscle cells, the initial pathogenic step in neointimal proliferation [5, 9, 17]. Thus, it is tempting to speculate that pressure-related endothelial cell damage possibly prompted proliferation of medial smooth muscle cells in the performed organ culture. However, we cannot exclude other underlying mechanisms.

Our findings on pressure-related endothelial cell loss and neointimal proliferation were compared with endothelial damage at baseline. Heterogeneity in SVG quality is common. Pre-existing impaired endothelial cell coverage and neointimal proliferation are 'the real world' of SVG quality in patients referred for CABG. Indeed, the prevalence of vascular risk factors such as hypertension, diabetes mellitus and a history of smoking was high in the patients included in the present study. Furthermore, surgical manipulation during harvesting might cause considerable damage to SVG endothelium. These two factors could be responsible for the endothelial cell loss of 20% and the pre-existing neointimal proliferation observed at baseline in SVGs in the present study. Roubos *et al.* [14] report a 7% endothelial cell loss in a non-distended control group. Unfortunately, the study of Weiss *et al.* [15] contains no data on baseline endothelial cell damage [15].

The long pressure exposure times (30 min) used in this experimental model do not reflect the real situation during competence testing, in which SVGs in general experience several intraluminal peak pressures of variable magnitudes over a variable time. However, no comprehensive data address the significance of the pressure exposure time in relation to pressure magnitude for the extent of the resulting endothelial damage. Which factor is more profound for pressure-related endothelial damage is unknown: the pressure magnitude, the constant pressure, the rising pressure, the velocity of the rising pressure or the total pressure exposure time. We hypothesize that the weighting of these factors might even differ in acute and long-term endothelial damage. Most interestingly, our results on the extent of acute endothelial cell loss were similar to those of previous studies with considerably different pressure exposure times [14, 15]. This could possibly indicate that for the acute disruption of the endothelium, the rapid increase in pressure and the pressure magnitude are more relevant than the pressure exposure time. The same might not necessarily be true in neointimal proliferation with its complete restructuring of the intima over time. Persistent pressure might be a constant trigger. Further studies on the effect of pressure exposure times and possibly the velocity of pressure increase are warranted for a better understanding of pressure-related damage. However, despite the long pressure exposure times used in our study, the results clearly indicate the relationship between intraluminal pressure and the ensuing endothelial cell loss and neointimal proliferation.

When interpreting our findings, it has to be considered that our results are based on an experimental model that only partially reflects the 'real life' of SVG handling during CABG. Further complexity is added by the fact that the 'real life' of SVG handling is not standardized. In daily routine, the pressure exposure courses vary broadly, as do the solutions used for intraoperative preservation and competence testing and the harvesting techniques. All these factors were shown to influence endothelial damage [10, 11, 14, 15, 18]. Organ culture is an established

model for studying neointimal proliferation [11, 12]. However, it is an *ex vivo* model excluding several other factors influencing the development of neointimal proliferation, such as blood running through the veins, arterial pressure head at the aorto-SVG anastomosis and the time elapsed between harvesting and anastomosis of SVG [5].

In summary, we observed a sharp contrast between high peak distension pressures routinely reached during competence testing of SVGs in CABG and low pressure ranges causing significant acute and, most importantly, long-term endothelial damage *in vitro*. The initial damage from uncontrolled pressure application during competence testing might substantially contribute to limited graft patency over the years. Monitoring and controlling the distension pressures applied during competence testing seems to be mandatory.

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