Human internal thoracic artery grafts exhibit severe morphological and functional damage and spasmic vasomotion due to oxidative stress

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The internal thoracic artery (ITA) is the first choice for myocardial revascularization, but atherosclerotic lesions and perioperative vasospasm may still limit its functionality. Oxidative stress via the peroxynitrite – poly-(ADP-ribose) polymerase (PARP) cascade plays an important role in the pathogenesis of impaired vascular tone via endothelial injury. We aimed to investigate and describe the histology, PARP activation and functionality of ITA grafts and to assess the possible beneficial effect of PARP-inhibition.

Material/Methods: ITA specimens from 47 patients (26 men, mean age 66.2±1.7 years) who underwent coronary bypass surgery were processed for histological and immunohistochemical studies for oxidative stress and PARP activation, and were functionally tested with acetylcholine (ACh) and sodium nitroprusside (SNP) with or without PARP inhibition.

Results: The sections showed atherosclerotic alterations and oxidative and nitrosative stress were evidenced by positive 3-nitrotyrosine, 4-hydroxynonenal and PAR stainings. Functionally, 88.1% reacted to K-Krebs, 68.7% exhibited contraction after 1 μM phenylephrine, 29.9% exhibited relaxation to 30 μM Ach, and all precontracted segments relaxed to 30 μM SNP. High amplitude vasomotion was observed in 47.8% of the segments, which could be abolished by the application of 10 μM SNP. Incubation of the preparations with PJ34 did not improve endothelium-dependent vasodilation.

Conclusions: ITA grafts are severely damaged both morphologically and functionally in patients undergoing coronary artery bypass surgery, but PARP inhibition cannot improve their functional characteristics. The topical use of SNP to the ITA during the operation may improve vascular functions by dilating the vessels and eliminating the eventual spasmic vasomotion.

key words: internal thoracic artery • perioperative vasospasm • oxidative stress • PARP • vasomotion • vasodilation

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BACKGROUND

For coronary artery bypass graft (CABG), the primary choice is the internal thoracic artery (ITA), because of its better patency rate compared with other possible graft sources such as radial artery or saphenous vein [1–6]. The favorable short- and long-term patency of the internal thoracic artery is believed to be related to its better physiologic properties, particularly to the better preserved endothelial function [7,8]. Even then, atherosclerotic lesions and perioperative vasospasm of the ITA may decrease blood flow and consequently contribute to early myocardial ischemia, increasing perioperative morbidity and mortality [9,10]. To overcome perioperative spasm, vasodilators are often administered during the operation; however, guidelines for their use are not clearly defined and need further investigation [11–13]. In the traditional harvesting technique, ITA is prepared as a pedicle – the distal part is clipped, cut, and covered with a vasodilator-soaked cloth until anastomosis is performed. Immunohistochemical analysis has shown that this approach disrupted the integrity of the endothelia of the clipped arteries and decreased nitric oxide production, disturbing the balance between vasoconstrictor and vasodilator mechanisms, potentially leading to spasmic vasomotion [14]. Spasmic high-amplitude vasomotion has been described in several vascular beds and it manifests impaired regulation of vascular tone, which could result in spasm and inadequate blood supply to the tissues [15]. This phenomenon has not yet been taken into consideration in the topical treatments of grafts during coronary artery bypass surgery.

Although internal thoracic artery has lower incidence of atherosclerosis than other arterial grafts [4,16], the presence of calcification and other atherosclerotic manifestations can be found in ITA grafts as well [17]. Oxidative and nitrosative stress play an important role in the pathogenesis of atherosclerosis [18–20]. Elevated levels of oxidative DNA-damage have been shown in atherosclerotic plaques [21]. A major pathway by which free radicals induce cell damage is the peroxynitrite – poly-(ADP-ribose) polymerase (PARP) cascade. PARP is an energy-consuming nuclear enzyme, which becomes activated in response to DNA single-strand breaks. As a result, NAD^+ and ATP levels decrease, resulting in cell dysfunction and cell death via the necrotic route [22]. PARP activation was demonstrated to have a role in the development of atherosclerosis [23]. Impaired endothelial function is a result of the atherosclerotic progress and the possibility of reversing already developed endothelial dysfunction via inhibition of PARP was shown in murine models of atherosclerosis [24,25]. Endothelial function of ITA graft could be additionally impaired during the surgical procedure when additional oxidative stress and consequential PARP activation might damage the graft. The current topical treatments during the surgical procedure do not address this possibility.

The aims of the present work were: (1) to study the morphology and assess the functionality of ITA grafts; (2) to investigate spasmic vasomotion of the specimens; (3) to assess oxidative and nitrosative stress and PARP activation using immunohistochemistry; and (4) to test the hypothesis that PARP inhibition improves ITA graft functionality.

MATERIAL AND METHODS

Human tissue collection

Nonskeletonized segments of human internal thoracic arteries were obtained from 47 patients who underwent coronary bypass surgery (26 men, mean age 66.2±1.7 SEM years, BMI: 28.6±0.8 SEM; 26 had hypertension, 21 had diabetes mellitus). As expected, patients were on a wide range of cardiovascular drug treatments including statins, nitrates, β-blockers and calcium channel antagonists. The investigation was approved by the local institutional ethics review committee (TUKEB 8/2004.), and informed consent was obtained from all subjects.

Histology and immunohistochemistry

ITA specimens from 29 patients were immediately fixed in 4% buffered formalin, and paraffin sections were prepared. Sections were either stained with hematoxylin and eosin for histological analysis or processed for immunohistochemistry and treated with 0.6% H_2O_2 in methanol to quench endogenous peroxidase activity and microwaved in 0.2 M citrate buffer (pH 3.0) to retrieve antigenic epitopes. After blocking in 1.5% normal goat serum, the nitrosative stress marker anti-3-nitrotyrosine antibody (3-NT; polyclonal; 1:80; Upstate Biotechnology, Lake Placid, NY, USA), the lipid peroxidation marker anti-4-hidroxynonenal antibody (4-HNE; monoclonal; 1:100; Oxis International Inc., Portland, OR, USA) or an anti-poly-ADP-ribose antibody (PAR; polyclonal; 1:100; Calbiochem, San Diego, CA, USA) for the assessment of PARP activation were applied. A biotinylated secondary antibody and the ABC method were used to visualize specific staining using 3, 3'-diaminobenzidine-tetrahydrochloride (DAB) and H_2O_2 as substrate (Vector, Laboratories, Burlingame, CA, USA). Immunohistochemical slides were counterstained with Gill's hematoxylin (Accustain, Sigma Diagnostics, St. Louis, MO, USA).

Functional studies

The remaining 18 specimens were stored in ice-cold (4°C) Krebs' solution composed of $CaCl_2$ 1.5 mM, $MgSO_4$ 1.2 mM, NaCl 118 mM, $NaHCO_3$ 14.8 mM, KCl 4.6 mM, NaH_2PO_4 1.2 mM, glucose 11.1 mM and were processed within 2 hours after removal. The vessels were cleaned of all fat and adherent connective tissue in a Petri dish, cut to 3 mm-long ring segments, and mounted in organ chambers filled with warmed (37°C) and oxygenated (95% O_2, 5% CO_2) Krebs' solution. Isometric tension was measured with isometric force transducers (HBM, Q11), which were connected to a transducer–amplifier (HBM, MGA II) and recorded on paper (Kipp Zonen, BD300). A tension of 1 gram was applied and the rings were equilibrated for 60 minutes. Fresh Krebs was provided at 20-min intervals. After equilibration, the contractile response of arterial rings to a depolarizing solution of a modified Krebs' solution enriched in K^+ (124 mM) was first tested to evaluate their functional integrity. For the measurement of endothelial and smooth muscle functionality, rings were precontracted with phenylephrine (Phe, 10^{-6} M) and then dose–response curves (10^{-8} M – 3×10^{-5} M) to acetylcholine (ACh) and sodium-nitroprusside (SNP)

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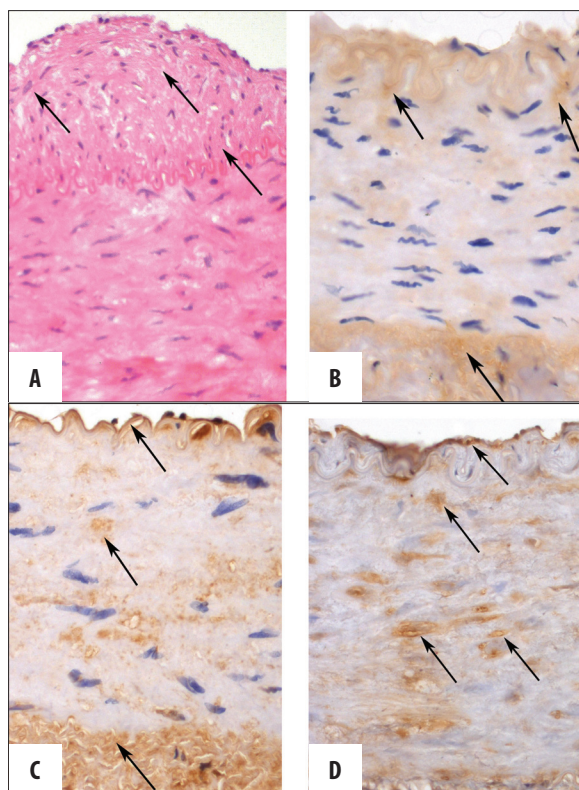
Figure 1. Histological and immunohistochemical results (40×
magnification). (**A**) Internal thoracic artery segment
showing intimal thickening, smooth muscle cell migration,
foam-cell formation (arrows). Oxidative and nitrosative
stress were evidenced by the positive 3-nitrotyrosine (**B**)
and 4-hydroxynonenal (**C**) staining. PARP activation (**D**)
was detected in all of the investigated segments (arrows
indicate specific stainings).

Figure 2. Functional characteristics of internal thoracic artery ring
segments. (**A**) Out of 67 segments 59 (88.1%) reacted
to K-Krebs, 46 (68.7%) exhibited contraction after 1 µM
phenylephrine. All precontracted segments relaxed to
SNP, but only 20 (29.9%) had any measurable relaxation
to Ach. Data expressed as percentage of all segments.
(**B**) Representative recording of the inhibitory effect of
10 µM sodium-nitroprusside on spasmic vasomotion.

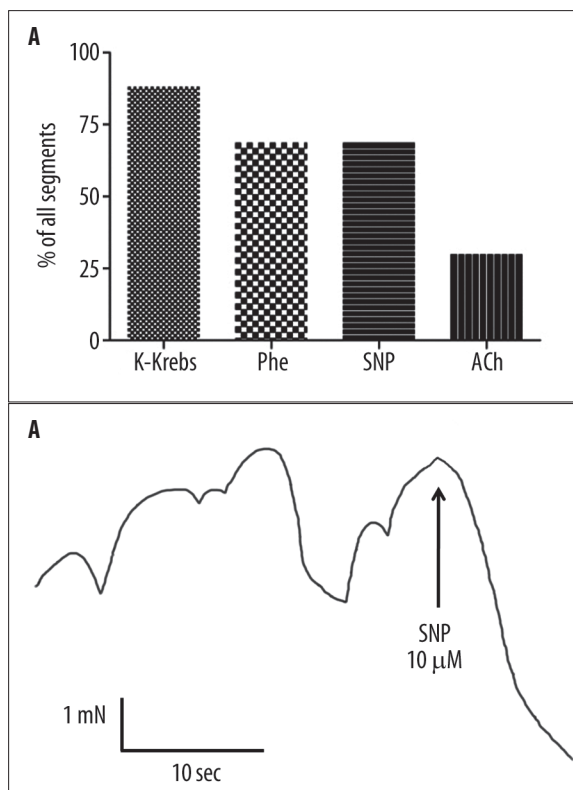were obtained. SNP (10 µM) was also used to test its effect
on vasomotion. To evaluate the possible beneficial role
of PARP-inhibition, 2 experimental groups were formed
from ring segments not exhibiting vasomotion, and dose-
response curves for Ach and SNP were developed on the
same segments before and 60 min after the application
of the phenanthridinone derivative PARP inhibitor PJ34
(1 µM) or its vehicle (saline).

Chemicals

PARP inhibitor PJ34 was obtained from Inotek
Pharmaceuticals Corporation (Beverly, MA, USA). All oth-
er chemicals were purchased from Sigma. Solutions of phen-
ylephrine, acetylcholine, sodium-nitroprusside and PJ34
were diluted to working concentration in saline on the day
of the experiment.

Statistical analysis

All values are reported as mean ±SEM. Statistical analysis
was performed using ANOVA and Dunnett post-hoc tests
using Graphpad Prism 4.03 statistical software. Values great-
er than 2 standard deviations outside the mean were con-
sidered statistical outliers. Probability values of $p < 0.05$ were
considered significant.

RESULTS

Histological and immunohistochemical findings

The sections showed signs of atherosclerosis, such as intimal
thickening, smooth muscle cell migration, foam-cell forma-
tion and calcification. Oxidative and nitrosative stress were
evidenced by positive 3-nitrotyrosine and 4-hydroxynonenal
staining, and the subsequent PARP activation was detected
in all of the investigated segments (Figure 1).

Functional characteristics of ring segments

The vasoactive function of the ring segments was very heter-
ogenous even among preparations from the same patient.
A total of 67 ring segments were cut from the specimens
and 59 (88.1%) reacted to K-Krebs, 46 (68.7%) exhibited
contraction after 1 µM phenylephrine, and all of these re-
laxed to 30 µM SNP. Regarding endothelial-derived vaso-
relaxation, only 20 ring segments (29.9%) showed at least
20% relaxation to 30 µM Ach (Figure 2A). High amplitude
vasomotion, an early sign of spasm, was observed in 32 out
of 67 ring segments (47.8%). These large-scale patterns of
vasomotion were terminated by 10 µM sodium-nitroprus-
side (Figure 2B).
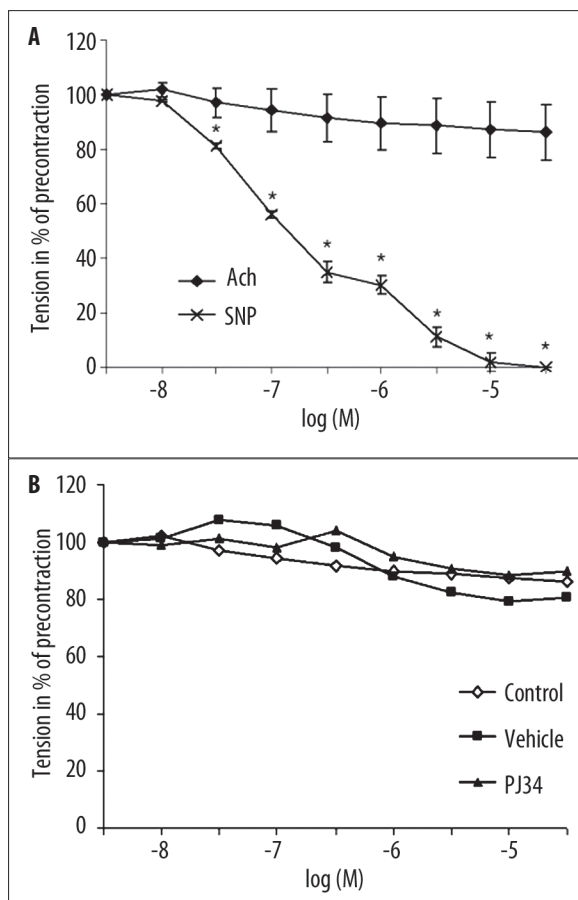
Figure 3. (**A**) Vessels precontracted with phenylephrine showed only a modest dilation to 30 μM Ach, while these vessels readily reacted to the endothelium-independent vasodilator sodium-nitroprusside at 10 μM. (**B**) Incubation of the preparations with 1 μM PJ34 PARP inhibitor did not improve vasodilation to Ach in our experimental conditions. Data expressed as mean±SEM (n=5 patients/group). For clarity, SEM values are not shown when no significant difference was found.

Vasodilatory potential and the effect of PARP inhibition

From the group not exhibiting vasomotion, 14 ring segments from 5 patients were precontracted with 1 μM phenylephrine and showed only 12.7±10.1% dilation to the highest (30 μM) dose of Ach, while these vessels completely relaxed to the endothelium-independent vasodilator sodium nitroprusside at 10 μM (Figure 3A). Incubation of the segments with 1 μM PJ34 PARP inhibitor or saline for 60 min did not improve vasodilation to Ach in our experimental conditions, as the maximal achieved relaxations were 11.4±13.6% and 19.2±12.8%, respectively (Figure 3B). The PARP inhibition did not affect the SNP-induced complete vasorelaxation.

DISCUSSION

Our study examined the histological and functional properties of internal thoracic artery grafts and investigated the effects of PARP inhibition. ITA segments showed signs of atherosclerosis, functional damage and SNP sensitive spasmic vasomotion. Marked oxidative and nitrosative stress and

PARP activation were detected in all of the investigated segments, but PARP inhibition did not improve the impaired vasodilatory capacity of the graft segments in our model.

Our histological results indicate that the majority of sections showed moderate to severe atherosclerotic modifications in the vascular wall, which is differs from the common presumption that ITA grafts are more preserved from atherosclerotic damage [4,16]. ITA segments exhibited intimal thickening, smooth muscle cell migration, macrophage accumulation and foam-cell formation, which are characteristics of atherosclerotic lesions.

Oxidative and nitrosative stress were evidenced by the positive 3-nitrotyrosine and 4-hydroxynonenal staining. 3-nitrotyrosine is a result of the nitration of tyrosine moieties by peroxynitrite, the product of NO and superoxide, therefore it serves as a long-term indicator of nitrosative stress-mediated protein modifications [26]. 4-HNE is a major lipid peroxidation product formed during oxidative stress; it promotes apoptosis and has a role in stress-mediated signaling [27]. Due to the oxidative and nitrosative stress evidenced by the 3-NT and 4-HNE stainings, PAR accumulation, and hence PARP activation, was detected in all of the investigated segments.

The vasoactive function of the ring segments was very heterogeneous even among preparations from the same patient. This result could be due to the fact that atherosclerotic lesions are not equally distributed along the arterial wall [28]. Vessels precontracted with phenylephrine showed only a limited dilation to the highest dose of Ach, while these vessels readily reacted to the endothelium-independent vasodilator sodium nitroprusside. The lack of dilation to Ach and the frequent occurrence of vasomotion indicate that the endothelial function of these vessels is severely damaged and the vascular regulatory mechanisms are impaired. The functionality of the vascular smooth muscle is better preserved, as the smooth muscles contract to phenylephrine and K-Krebs in most cases, and relax after the administration of the NO-donor SNP. As a limitation to this study it must be noted that although every measure was taken to avoid any injury to the vessels during harvest and storage, it cannot be completely ruled out that these disturbances may have had an effect on the vasoactive properties of the ring segments.

To the best of our knowledge this is the first report on high amplitude vasomotion in ITA grafts. Vasomotion has been described in several vascular beds and its physiological and pathophysiological importance is under intensive research [29–31]. Irregular high-amplitude vasomotion represents impaired regulation of the vascular tone, which could result in spasm and inadequate blood supply to the tissues [15,32]. The appearance of large-scale patterns in vasomotion reflects the inability of the vascular regulation to respond adequately to stimuli. Examining the characteristics of vasomotion could be a promising tool in diagnostic procedures, because changes in vasomotion are suspected to be the early signs of tissue hypoxia [33,34]. The disturbed balance of vasoconstrictors and vasodilators was shown to provoke vasomotion in the brain via thromboxane-2-receptors [32]. Although the regulation of the cerebral circulation and the skeletal circulation (ITA) differ substantially,

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further studies investigating the role of these receptors in the development of perioperative spasm in the internal thoracic artery are warranted.

PARP inhibition did not improve endothelial-derived vasodilatory capacity in the current experimental setting. The reason for this outcome could be that the segments used in this study were from relatively elderly patients (mean age 66.2±1.7 [SEM] years) compared to other studies [35,36], who suffered from atherosclerosis and other metabolic or systemic diseases. These conditions (e.g., diabetes, hypertension, hyperlipidemia, obesity) further damaged the vasodilatory capacity of the arteries by inducing oxidative stress in the endothelium. Previous studies have shown that there was a strong correlation between ITA atherosclerosis and age [17], which might explain the failure of PARP inhibition to improve the endothelial function of our segments from elderly patients, although the possibility of such an effect was demonstrated in animal models [24,25]. It might be possible that a similar approach in younger patients with limited additional diseases could be successful, and this possibility needs further investigation. In summary, we found that: (1) internal thoracic arteries showed marked atherosclerotic signs due to oxidative and nitrosative stress; (2) the vessels are functionally impaired; (3) sodium-nitroprusside improved vascular functions by dilating the vessels and eliminating spasmic vasomotion; and (4) PARP inhibitor treatment failed to improve endothelial dysfunction.

CONCLUSIONS

Based on our results, we conclude that internal mammary artery grafts are severely damaged in elderly patients and PARP inhibition cannot improve their functional characteristics. It is beneficial and recommended to apply sodium-nitroprusside to the ITA during coronary artery bypass graft surgery to achieve a more dilated graft and to overcome the eventual perioperative spasmic vasomotion.

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