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European Journal of Cardio-thoracic Surgery 28 (2005) 551–557

EUROPEAN JOURNAL OF CARDIO-THORACIC SURGERY

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Skeletonization of internal thoracic artery affects its innervation and reactivity

Marek Andrzej Deja[a,*], Krzysztof S. Gołba[b], Marcin Malinowski[a], Stanisław Woś[a], Maciej Kolowca[a], Jolanta Biernat[b], Maciej Kajor[c], Tomasz J. Spyt[d]

[a]2nd Department of Cardiac Surgery, Medical University of Silesia, Ul. Ziołowa 47, 40-635 Katowice, Poland
[b]2nd Department of Cardiology, Medical University of Silesia, Ul. Ziołowa 47, 40-635 Katowice, Poland
[c]Department of Pathology, Medical University of Silesia, Katowice, Poland
[d]Department of Cardiothoracic Surgery, University Hospitals of Leicester, Glenfield Hospital, Leicester, UK

Received 30 March 2005; received in revised form 17 June 2005; accepted 28 June 2005; Available online 26 August 2005

Abstract

Objective: The studies showing the superior characteristics of ITA graft and its impact on the clinical results of coronary artery surgery were performed with ITA harvested almost exclusively as a pedicle. This study assesses the impact of ITA skeletonization on its innervation and reactivity. **Methods**: Segments of skeletonized and non-skeletonized ITA were stained with antibodies against protein S-100 to look for the presence of sympathetic nerve fibers. The functional studies were performed on segments of discarded human pedicled ITA that were divided into two 3 mm rings, one skeletonized and another non-skeletonized. We compared concentration–effect relationships for the contraction to norepinephrine and endothelium-dependent relaxation to acetylcholine and bradykinin, as well as endothelium-independent relaxation to sodium nitroprusside in skeletonized and non-skeletonized segments of the same ITA. **Results**: Skeletonized ITA was devoid of protein S-100 positive nerve fibers. It contracted stronger (maximal response 37.0 ± 2.04 vs. 25.4 ± 1.83 mN ($P<0.001$)) and was twice as sensitive to norepinephrine: pD_2 6.03 ± 0.10 vs. 5.70 ± 0.12 ($P=0.035$). The endothelium-dependent relaxation responses did not differ between skeletonized and non-skeletonized ITA rings. The skeletonized ITA rings appeared over 10 times more sensitive to sodium nitroprusside: pD_2 6.66 ± 0.20 vs. 5.59 ± 0.37 ($P=0.012$)—potency ratio 11.61. The maximal responses did not differ significantly: 112.0 ± 6.71 vs. $129.4\pm16.4\%$ ($P=0.33$). **Conclusions**: Skeletonization results in sympathectomy of ITA. It has no effect on endothelium-dependent relaxation but increases reactivity of ITA to norepinephrine. This augmented response to α-agonist is small, in comparison with over a ten-fold increase in sensitivity to sodium nitroprusside. Pedicled and skeletonized ITA are functionally significantly different vessels when studied in vitro.

Keywords: Internal thoracic artery; Sympathectomy; Endothelium-dependent relaxation; Endothelium-independent relaxation

1. Introduction

During coronary artery by-pass surgery the internal thoracic artery (ITA) may be harvested either as a pedicle together with concomitant veins, lymphatics, sympathetic plexus and internal thoracic fascia or skeletonized free of all surrounding tissue as originally used by Grondin in 1970s [1] and described by Keeley in 1987. The latter technique allows for longer availability of the graft, hence opportunity for more distal coronary artery anastomoses and easier sequential grafting [2]. The increased early blood flow through the skeletonized ITA has also been implied, as a factor decreasing the risk of hypoperfusion syndrome [3]. The chest wall injury is minimized, and the sternal blood supply is preserved [4] which is particularly important in diabetic patients and those receiving both ITAs. Better postoperative respiratory function has also been reported [5]. The disadvantage is that skeletonization is time consuming and technically more demanding, increasing the chances of ITA injury. Nevertheless, gentle skeletonization has been shown to preserve internal thoracic artery morphological integrity [6]. Moreover, we have previously reported that the endothelium-mediated relaxation to acetylcholine in arteries harvested with both techniques is similar [7].

The studies showing the superior characteristics of ITA graft and its impact on the clinical results of coronary artery surgery were performed with ITA harvested almost exclusively as a pedicle [8]. However, biological properties of skeletonized ITA might be in some aspects different from pedicled ITA. It is believed that ITA skeletonization results in its sympathectomy. Still, it has not been shown conclusively. In fact this assumption was challenged recently [9].

It might also be appropriate to ask, whether vascular reactivity of skeletonized ITA differs from the pedicled ITA.

* Corresponding author. Tel./fax: +48 32 2526093.
E-mail address: narizol@slam.katowice.pl (M.A. Deja).

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doi:10.1016/j.ejcts.2005.06.037

After all, if not the sympathetic plexus influence, the periarterial tissue paracrine activity [10] might be of importance.

2. Materials and methods

The study was performed on isolated segments of left ITA discarded after the conduit had been trimmed to the length necessary for grafting. The Local Research Ethics Committee agreed to the use of human tissue for the experimental work. The study arterial segments were obtained from patients undergoing surgery for stable coronary artery disease.

The ITA harvesting method in our institution has been described previously [7]. In brief, when harvested in pedicle the internal thoracic fascia was incised with electrocautery along both sides of the ITA approximately 0.5 cm away from concomitant veins. The flap of fascia, muscle, and fat tissue containing the ITA with concomitant veins was dissected with electrocautery, working from its distal to proximal end, and ligating the major ITA branches with Ligaclips (Ethicon, Somerville, NJ). When skeletonizing, the left pleura was dissected laterally so that the LITA and its concomitant veins could be seen along their course. The internal thoracic fascia was incised just medial to the LITA in the second intercostal space, where it is less adherent to the chest wall. The skeletonization of the LITA was performed from this point distally leaving both concomitant veins at the chest wall. Apart from cutting the fascia, most of the dissection was blunt. The tributaries were ligated tangential to the vessel wall and distally and were divided with scissors to avoid heat injury. After the LITA had been harvested to its bifurcation level, the concomitant vein was divided at the first intercostal space, and the skeletonization was performed proximally up to the subclavian artery.

2.1. Histological examination

We studied histologically the segments of 10 ITA harvested in pedicle and 10 harvested skeletonized.

We divided the segments obtained from pedicled ITA and skeletonized half of them in laboratory, before fixing with 4% paraformaldehyde. These in vitro skeletonized segments were compared with in vivo skeletonized segments by blinded histopathologist, using hematoxylin–eosin staining to verify the assumption that the two were practically indiscernible.

Next, we assessed integrity of sympathetic plexus in arterial wall. Although sympathetic nerve fibers are unmyelinated [11], they contain Schwann cells, which can be stained with antibodies against protein S-100 [12]. Thus, we looked for them in 10 segments of skeletonized ITA and 10 segments of non-skeletonized ITA using anti S-100 antibodies provided by DakoCytomation Norden A/S (Glostrup, Denmark).

2.2. Isolated organ study

All the arteries were harvested pedicled using electrocautery. The discarded segments of ITA were placed in the ice-cold calcium free modified Krebs–Henseleit solution and transferred to the laboratory. The vessels were then divided into 3 mm long segments. Half of them were dissected free of the surrounding tissue (skeletonized). The two arterial rings from the same patient, one skeletonized and one non-skeletonized were studied simultaneously.

The arterial rings were suspended on stainless steel wire hooks in the organ bath chamber filled with oxygenated (95%O_2, 5%CO_2) Krebs–Henseleit solution of the following composition: NaCl, 118.0; KCl, 4.70; $CaCl_2$, 2.52; $MgSO_4$, 1.64; $NaHCO_3$, 24.88; KH_2PO_4, 1.18; glucose, 5.55; sodium pyruvate, 2.0 [mM] (pH 7.4). The temperature was maintained at 37 °C. The Schuler isolated organ bath (Hugo Sachs Elektronik (HSE); March-Hugstetten, Germany) was used. Vessel wall tension was measured with isometric force transducer F 30 (HSE) with bridge amplifier (HSE) and recorded using the Graphtec WR 3310 linear recorder (HSE).

After the short period of initial incubation the vessel wall tension and diameter were normalized in a standardized procedure as described by Mulvany and Halpern [13]. This way every vessel ring was set to the 90% of diameter it would have had in vivo, when relaxed and under the transmural pressure of 100 mmHg using the Laplace law; $P=2T/d$. After normalization the vessel was left for 30 min to stabilize, during which period the tissue was thoroughly washed.

2.3. Experimental protocol

Initially, the artery was contracted with 10^{-5} M norepinephrine. This concentration was found to cause submaximal contraction in previous experiments. The precontracted artery was subjected to 10^{-5} M acetylcholine next to test for the presence of functioning endothelium. The artery was washed until the wall tension returned to the initial level.

Next, the artery was gradually contracted with norepinephrine starting from 10^{-9} M and rising in negative logarithm half molar cumulative steps up to 10^{-4} M to establish the concentration–effect relationship. The artery was thoroughly washed for at least 40 min to return the vessel wall tension to the basal level and the concentration-response to one of the vasodilators was studied next. To do this the arterial ring was first precontracted with 10^{-5} norepinephrine and than gradually relaxed with either acetylcholine or bradykinin or sodium nitroprusside, all starting from 10^{-9} M and rising in negative logarithm half molar cumulative steps up to 10^{-4} M. Acetylcholine and bradykinin were used to study endothelium-dependent and sodium nitroprusside to study endothelium-independent relaxation. Only two concentration–effect relationships were studied in every experiment. The contraction response was always studied first. In experiments in which only relaxation responses were studied the endothelium-dependent relaxation was studied first.

We obtained 15 paired (skeletonized and non-skeletonized) concentration–effect curves for every substance used.

The following substances were used: (−)arterenol bitartrate, acetylcholine chloride, bradykinin triacetate, sodium nitroprusside (Sigma-Aldrich Corp., St Louis, MO).

The artery contraction was measured as an increase in the vessel wall tension above the resting tension. The relaxation was assessed as the decrease in the wall tension from the precontracted level and expressed as a percentage of the contraction obtained with norepinephrine.

The relaxation responses at each concentration level were presented as a mean±SEM and compared using the paired *t*-test. To test whether the skeletonization influenced the reactivity of ITA to the given substance we used two-way analysis of variance with concentration level as one factor and skeletonization as another. The concentration-effect relationships were obtained from a regression analysis to the general logistic equation of Michaelis and Menten (1913)

$$E = \frac{E_{max} D^n}{D^n + K_D^n}$$

where E is effect, E_{max} is maximal effect, D is concentration, K_D is drug-receptor complex dissociation constant equal to the concentration causing half-maximal effect (EC_{50}) and n is the Hill coefficient. The estimated EC_{50} was subsequently log transformed and presented as $pD_2 = -\log(EC_{50})$. This was compared using Student's *t*-test. In all instances of statistical analysis, the $P<0.05$ was considered significant.

3. Results

3.1. Histological examination

The arterial segments of in vitro and in vivo skeletonized ITA were virtually indistinguishable under microscope. The arterial wall was almost intact with very little adjacent tissue. We found no hematoma or dissection in the media. The endothelial lining was well preserved.

When comparing the presence of protein S-100 positive fibers in segments of skeletonized and non-skeletonized ITA (Fig. 1), we found at least 1 (average 1.5±0.7, median 1, range 1-3) fiber in the vicinity of ITA in every non-skeletonized ITA preparation and we failed to find the nerve fiber in all but one of 10 studied skeletonized ITA preparations ($P<0.001$).

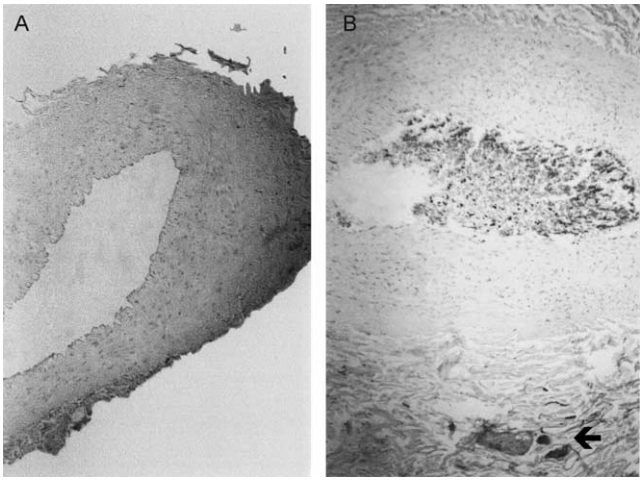

Fig. 1. The immunohistochemical study of skeletonized (A) and non-skeletonized ITA (B). The preparations were stained with antibodies against protein S-100 to look for the nerve fibers (arrow). Magnification 50×.

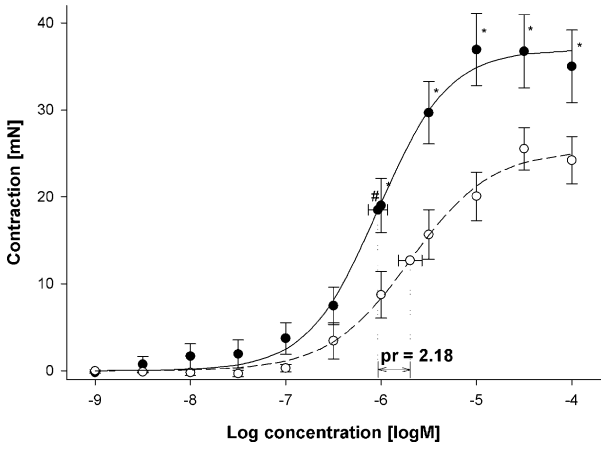

Fig. 2. Concentration-response curves (logistic regression lines) for contraction to norepinephrine. Symbols represent mean effect at the given concentration±SEM. EC_{50}±SEM is depicted as well. —●— skeletonized ITA, ---○--- non-skeletonized ITA. *$P<0.05$ skeletonized vs. non-skeletonized as per paired *t*-test; pr, potency ratio.

3.2. Isolated organ study

The skeletonized ITA contracted stronger to norepinephrine in comparison with non-skeletonized ITA; maximal response of 37.0±2.04 vs. 25.4±1.83 mN, respectively ($P<0.001$). Furthermore, the skeletonized ITA was twice as sensitive to norepinephrine: pD_2 6.03±0.10 vs. 5.70±0.12 ($P=0.035$) with potency ratio of 2.18 (Fig. 2).

The endothelium-dependent relaxation responses did not differ between skeletonized and non-skeletonized ITA rings. Both groups were equally sensitive to bradykinin: pD_2 6.96±0.30 vs. 6.76±0.34 ($P=0.65$) (Fig. 3). The maximal relaxation equaled 35.7±3.61 vs. 36.9±3.90% ($P=0.81$). Similarly sensitivity to acetylcholine was equal in both groups: pD_2 7.10±0.12 vs. 7.12±0.14 ($P=0.92$) (Fig. 4). We

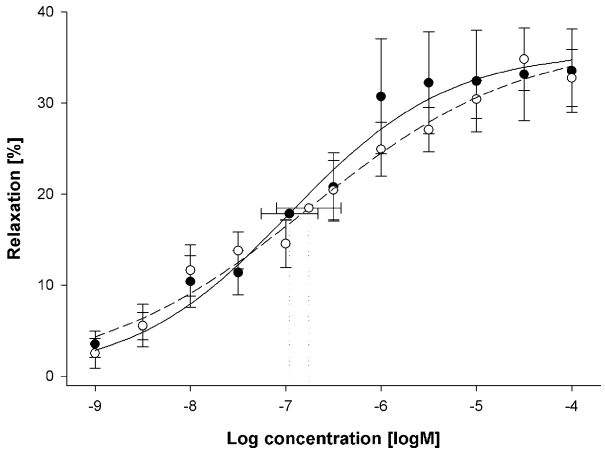

Fig. 3. Concentration-response curves (logistic regression lines) for relaxation to bradykinin. Symbols represent mean effect at the given concentration± SEM. EC_{50}±SEM is depicted as well. —●— skeletonized ITA, ---○--- non-skeletonized ITA.

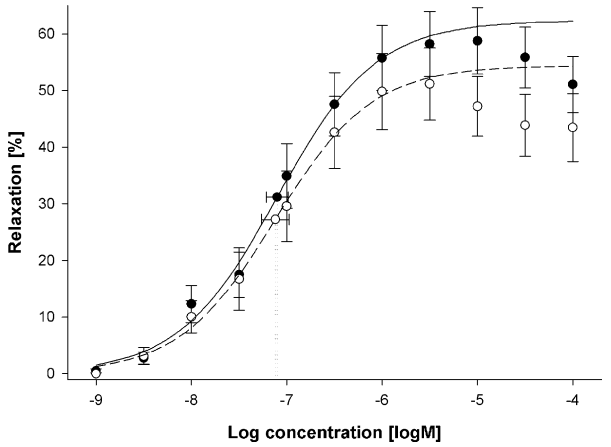

Fig. 4. Concentration–response curves (logistic regression lines) for relaxation to acetylcholine. Symbols represent mean effect at the given concentration ± SEM. EC_{50} ± SEM is depicted as well. —●— skeletonized ITA, ---○--- non-skeletonized ITA.

measured no significant difference in maximal relaxation response. However, the maximal response estimated by nonlinear regression analysis, tended to be slightly higher for skeletonized than non-skeletonized arteries: 62.4 ± 2.72 vs. 54.4 ± 2.97% ($P = 0.049$). Similarly, the two-way analysis of variance with acetylcholine concentration level as one factor and the presence or absence of surrounding tissue as another showed that skeletonization changed tissue responsiveness to acetylcholine significantly ($P = 0.034$). This was not the case for the reactivity to bradykinin ($P = 0.55$).

The relaxation to sodium nitroprusside was significantly affected by skeletonization (Fig. 5).

The skeletonized ITA rings appeared over 10 times more sensitive to sodium nitroprusside: pD_2 6.66 ± 0.20 vs. 5.59 ± 0.37 ($P = 0.012$)—potency ratio 11.61. The maximal responses did not differ significantly: 112.0 ± 6.71 vs. 129.4 ± 16.4% ($P = 0.33$).

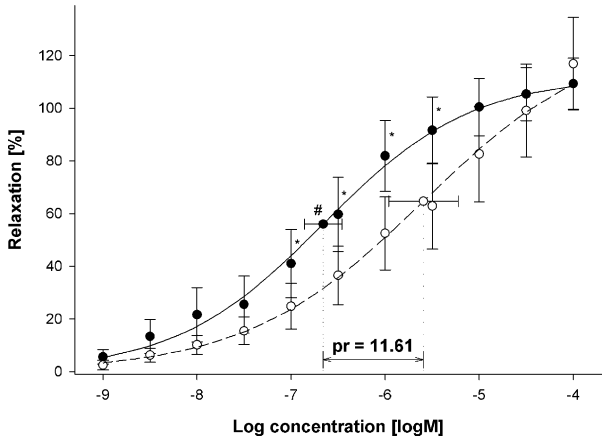

Fig. 5. Concentration–response curves (logistic regression lines) for relaxation to sodium nitroprusside. Symbols represent mean effect at the given concentration ± SEM. EC_{50} ± SEM is depicted as well. —●— skeletonized ITA, ---○--- non-skeletonized ITA. *$P < 0.05$ skeletonized vs. non-skeletonized; pr, potency ratio.

4. Discussion

It is routine in isolated tissue experiments to remove all surrounding tissue from vascular rings studied. We previously studied the endothelium-dependent responses of the ITA segments harvested skeletonized and compared them with those harvested as a pedicle. We found no significant difference [7]. All specimens transferred to the laboratory were treated in a routine way, i.e. dissected free of adjacent structures. We were able to show that the endothelial function was equally satisfactory irrespective of the surgical technique used, but no further conclusions were possible studying arterial segments that were all 'skeletonized' in the laboratory.

In the present paper we describe the reactivity of the rings of ITA that were all harvested as a pedicle, and subsequently half of them were skeletonized in the laboratory. In every experiment a pair of skeletonized and non-skeletonized 3 mm long rings obtained from the same ITA were studied. This allowed for a pair-wise comparison of reactivity. We believe it is important even bearing in mind that the ITA skeletonized ex vivo may be not identical to the one skeletonized in the operating room. To avoid this weakness one would need to prospectively compare responses of ITAs harvested skeletonized and pedicled from different patients. Differences between patients, like their pharmacotherapy or comorbidities (diabetes, hypertension, etc.) would make results difficult to interpret.

The histological analysis by blinded histopathologist confirmed that the ITA segments skeletonized in vitro were microscopically indistinguishable from those skeletonized in vivo. It allows, with due caution, for extrapolation of the results of present study to the clinical situation.

The most important finding of histological part of our study is that ITA skeletonization results in its denervation. This is not surprising but it confirms that skeletonization results in sympathectomy. Therefore, at least some of the changes observed in the reactivity of skeletonized and non-skeletonized ITA might be attributed to the removal of sympathetic plexus.

Sympathetic nerve fibers are all unmyelinated and located in the adventitia [11]. They can be identified with antibodies against S-100 protein present in Schwann cells [12], which in case of unmyelinated fibers simply encapsulate the axon [14]. Our findings are disparate with those of Gaudino et al. [9] who found no difference in the number of S-100 protein positive fibers between skeletonized and pedicled ITA segments. The authors admit that these fibers were present predominantly in the outer third of artery's adventitia. When comparing the microscopic photographs of the skeletonized artery from Gaudino's et al. and from our paper it becomes obvious that when skeletonizing ITA we left much less loose connective tissue in the tunica adventitia. It is therefore even more important to stress that blinded histopathologist (M.K.) could not differentiate under microscope in vivo and ex vivo skeletonized arterial segments. On the other hand, it shows that skeletonization may differ in the hands of different surgeons. It may be not possible, however, to assure that it is not close enough to spare

the sympathetic plexus in the outer third of adventitia along all the harvested ITA.

The sympathetic fibers finish with the abundant network of nerve endings excreting norepinephrine located adjacent to media and generally not entering it [11]. These nerves could be identified with antibodies against tyrosine hydroxylase and 160 kD neurofilament [9]. We made no attempt to stain them, as they are the endings of the very same fibers that we identified in more superficial layers of adventitia with anti S-100 protein antibody. Moreover, their location in the immediate vicinity of media makes their damage during skeletonization unlikely.

The results of isolated organ study showed some significant differences between skeletonized and non-skeletonized segments of the same ITA. The reactivity was different when the agents acting directly on the smooth muscle were applied. There was little difference when endothelium-dependent vasodilation was tested. Two-way ANOVA found that skeletonization influenced relaxation in response to acetylcholine but not to bradykinin. This can be explained by the fact that acetylcholine action is not entirely endothelium mediated. This effect is probably of no biological significance, as no difference could be shown when directly comparing pD_2, maximal relaxation, or the effects of particular concentrations of the agonist.

This unchanged endothelial function is very important for the graft function [15]. The result is interesting as there are some reports of adrenergic influence on nitric oxide production [16], as well as on the inhibition of endothelium-dependent relaxation by sympathectomy, including local periarterial sympathectomy [17,18]. The reports are, however, contradictory. If one of the major effects of ITA skeletonization is damage to the periarterial adrenergic plexus, then our results concur with reports showing that sympathectomy does not affect endothelium-dependent relaxation [19,20].

Endothelium-dependent responses probably contribute to long described ability of ITA to adapt its diameter in response to varying flow [21], the ability which is absent in saphenous vein graft. This ability maintains relatively high shear stress in ITA graft even in face of competitive flow, which in turn is supposed to ensure a longer patency [22]. Comparable endothelium-dependent relaxation in our study makes us hope that this adaptive behavior will be preserved in skeletonized ITA. However, some studies suggest that even in face of preserved endothelial function in sympathectomized artery, its contribution to flow regulation may be changed, i.e. diminished [19,20,23]. To find the answer to this very pertinent question one would need to perform an in vivo experimentation comparing the changes in skeletonized vs. pedicled ITA graft diameter in response to increased flow, due to e.g. tachycardia, similar to the one performed by Hanet et al. for ITA and saphenous vein grafts [21].

As already stated the difference in ITA reactivity in our experiments was significant in cases of substances acting directly on the smooth muscle and not on the intraluminal surface of the artery. Generally, the skeletonized artery was more sensitive to both norepinephrine and sodium nitroprusside. It is possible that it relates to the diffusion process. One might expect the diffusion of the agonist through the periarterial tissue to be more difficult in comparison with 'naked' skeletonized artery resulting in decreased responses. Indeed, the tension rise in response to any single norepinephrine concentration increase was generally much slower in non-skeletonized vs. skeletonized arterial ring (Fig. 6). We needed to wait longer for the response plateau after every concentration step when recording the concentration–response relationship for norepinephrine. We believe, however, that if the problem were merely one of physical diffusion, the final response although slower would be of the same magnitude. If the periarterial tissue were actively interacting with norepinephrine than the results would differ. A simple explanation might be the norepinephrine uptake by adrenergic plexus, which is absent in a skeletonized artery. This would account for both observed higher sensitivity and stronger maximal contraction in response to norepinephrine in skeletonized segments.

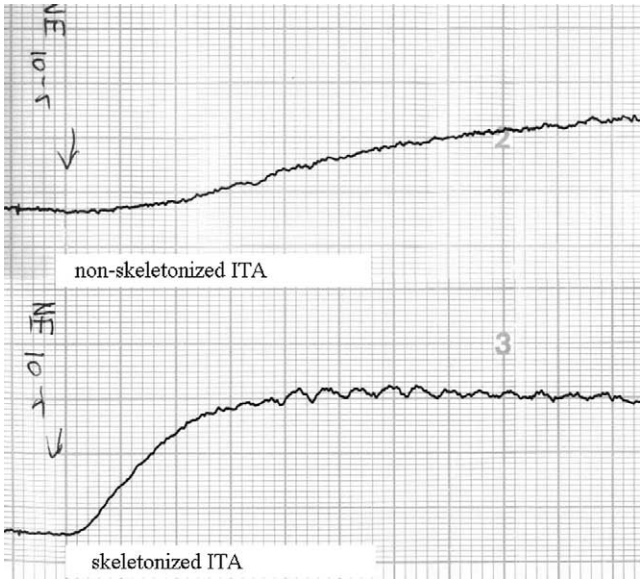

Fig. 6. The sample of the original recording showing the response to one of the concentrations of norepinephrine in non-skeletonized and skeletonized ITA ring.

Another probable explanation is that periarterial tissue possesses paracrine function. It has been shown recently that adventitia secrets ADRF (adventitium-derived or adipocyte-derived relaxing factor) of unknown nature which activates smooth muscle K^+ channels and has anticontractile properties [10]. Its release is Ca^{2+} dependent and does not depend on periarterial nerve endings [24]. Thus, ADRF release may explain observed results. This would mean that skeletonization might change ITA reactivity independent of putative sympathectomy. Whether ADRF truly plays a role in function of ITA requires further investigation.

Meanwhile, the increased contraction responses after sympathectomy have been long observed both in vitro and in vivo and ascribed to 'denervation' [25]. Furthermore, the increased sensitivity to norepinephrine may be of importance to the surgeon as the infusion of catecholamines is common in the early post coronary artery surgery period.

Similarly, the significantly increased sensitivity to sodium nitroprusside is probably associated with its interaction with periarterial tissue. An alternative explanation would be increased myocyte sensitivity to nitric oxide in skeletonized ITA. However, this would have significantly influenced the response to acetylcholine and bradykinin, unless the endothelium had been secreting over 10 times less nitric oxide in skeletonized ITA to exactly match for the rise in myocyte sensitivity. The latter seems rather unlikely. We cannot explain the nature of the interaction between periarterial tissue and sodium nitroprusside. This may have a clinical significance, as lower concentrations of myorelaxant were necessary to produce significant vasodilation in skeletonized ITA.

Our results have to be compared with in vivo reactivity of skeletonized and pedicled ITA that has recently been published [9]. The authors found no significant difference in ITA responses to serotonin, and isosorbide dinitrate injected intraluminally and to methylergometrine injected intravenously. However, only one concentration of every drug was studied and understandingly the concentration was lower in comparison with what can be applied in vitro. In spite of that and of lack of significant differences, the tendency to stronger contraction to α-agonist methylergometrine and stronger relaxation to isosorbide dinitrate in distal segments of skeletonized in comparison with pedicled ITA may be appreciated in the data of Gaudino et al. [9]. One obviously has to bear in mind that the reactivity in vitro may differ from in vivo as the substances studied act from both extraluminal and endoluminal side of the vessel wall in former and only from endoluminal side in latter situation.

In addition certain limitations of in vitro experiments have to be pointed. The most obvious is that ITA skeletonized immediately before organ bath studies is not the same as denervation in vivo an hour or a week earlier. There must be a certain time sequence for depletion of sympathetic nerve fiber terminals of norepinephrine and subsequent resetting of the receptors after denervation. We searched the literature extensively and failed to find the definite answer to what this time sequence was like in case of e.g. lumbar sympathectomy. Thus, reactivity might change, and almost certainly changes, not only from ex vivo to in vivo situation, but also in vivo with time passed from surgery. Additionally the innervation of the pedicle harvested ITA late after surgery has not been studied either. On the other hand, the skeletonized ITA, naked after surgery, becomes buried in surrounding mediastinal tissue and while healing undergoes neovascularization absent in pedicled graft [6]. This may lead to changes in reactivity as well.

In summary, bearing in mind all the limitations, our study revealed that skeletonization results in sympathectomy of ITA. It has no effect on endothelium-dependent relaxation but increases reactivity of ITA to norepinephrine. This augmented response to α-agonist is, however, small in comparison with over a 10-fold increase in sensitivity to sodium nitroprusside. The different function results either from sympathectomy, or from the removal of perivascular tissue paracrine activity, or from both.

We conclude that pedicled and skeletonized ITA differ significantly from their reactivity point of view when studied in vitro. As vasodilators are often applied to the outside of the artery during its preparation for grafting, higher sensitivity might be beneficial.

Whether described differences in both histological and functional characteristics of skeletonized and pedicled ITA have clinical ramifications may be difficult to establish and requires long-term follow-up studies.

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