Original article

N-acetylcysteine prevents neointima formation in experimental venous bypass grafts

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Background: Neointima formation, mainly characterized by smooth muscle cell proliferation, is an important cause of venous bypass graft failure. The therapeutic potential of the antioxidant *N*-acetylcysteine (NAC) to attenuate smooth muscle cell proliferation and neointima formation was examined *in vivo*. The effects of NAC on hyperoxia-induced venous smooth muscle cell (VSMC) cytokine production and proliferation were addressed *in vitro*.

Methods: Rats underwent autologous epigastric vein-to-femoral artery interposition grafting. Fourteen rats received oral NAC, and a similar control group received saline. Histomorphometric analysis was performed after 7 days or 3 weeks. Cytokine analysis and cell proliferation assay were performed in cultured human VSMCs after hyperoxic or normoxic exposure and NAC administration.

Results: NAC-treated rats displayed a threefold reduction in neointimal area, a sixfold reduction in stenosis rate, and a twofold reduction in VSMC proliferation after vein graft surgery. Incubation of VSMCs in 70 per cent oxygen stimulated the release of mitogenic inflammatory cytokines interleukin (IL) 6 and IL-8. Cytokine-rich medium from these VSMCs induced proliferation of normoxic VSMCs. NAC inhibited hyperoxia-induced cytokine release and VSMC proliferation.

Conclusion: NAC attenuated neointima formation and vein graft stenosis by reducing VSMC proliferation *in vivo*, and prevented hyperoxia-induced cytokine production and VSMC proliferation *in vitro*.

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Introduction

Implantation of autologous saphenous vein segments in the arterial circulation has been the treatment of choice for many years to bypass peripheral and coronary arterial obstructions. However, vein graft stenosis and occlusion due to neointima formation is a major problem resulting in unsatisfactory patency levels[1]. Intimal hyperplasia is characterized by medial smooth muscle cell proliferation and migration into the subintimal space, increased extracellular matrix deposition, and formation of a thick neointima. Arterial bypass conduits have superior patency rates to saphenous vein grafts[2]. This disparity has been attributed to differences in the structural, cellular and molecular composition of arterial and venous walls, resulting in altered adaptation to arterial haemodynamics[3,4], and different responses to atherogenic and mitogenic stimuli in arterial and venous grafts[5]. Recently, oxidative stress has also been related to differences in patency between vascular grafts[6].

Oxidative stress, defined as an imbalance between reactive oxygen species and endogenous antioxidants in the vascular tissue, may play an important role in the pathogenesis of cardiovascular diseases like atherosclerosis, hypertension and heart failure[6–8]. Reactive oxygen species, including hydroxide, hydrogen peroxide and oxygen, are generated by virtually all types of vascular cells[9]. Under

physiological conditions, the vasculature has antioxidant reserves to cope with the production of reactive oxygen species, excessive levels of which have been shown to induce endothelial dysfunction, with subsequent impairment of anticoagulant and anti-inflammatory properties, and smooth muscle cell activation[10].

Venous grafts exhibit higher oxidative stress than arterial grafts[11]. Oxidative stress in venous grafts may relate to surgical trauma, haemodynamic stress[12–14] or exposure of the venous segment to the oxygen-rich environment of the arterial circulation. The role of perceived hyperoxia in tissue remodelling is well appreciated in pulmonary arteries[15,16], as well as in the heart following ischaemia–reperfusion[17]. The effect of relative hyperoxia on venous smooth muscle cells (VSMCs) and its role in neointima formation in venous grafts have not been investigated.

The aim of the present study was to determine whether treatment with the oxygen free radical scavenger *N*-acetylcysteine (NAC) attenuated neointima formation in venous bypass grafts *in vivo*. The potential role of perceived hyperoxia and NAC on VSMC cytokine production and proliferation *in vitro* was also addressed.

Methods

In vivo

Experimental procedures were in accordance with the Dutch law on the use of laboratory animals and approved by the institutional Ethics Committee. Male inbred specific pathogen-free Lewis rats were obtained from the Department of Experimental Animal Service of the University of Maastricht. Experiments were performed in animals aged 12 weeks weighing 300–400 g. Rats were fed standard chow and tap water *ad libitum*. All surgical procedures were performed under general anaesthesia and using sterile techniques.

Rat venous graft model and drug treatment protocol

Autologous epigastric vein to common femoral artery end-to-end interposition grafts were constructed in rats as described previously[18,19]. Briefly, each animal was anaesthetized with an intraperitoneal injection of 60 mg/kg sodium pentobarbital. The common femoral artery was clamped, and a 3-mm segment excised from the centre of the clamped area. An 8-mm segment of the ipsilateral epigastric vein was harvested, gently irrigated with heparinized saline (100 units/ml), reversed as an interposition graft in the arterial defect, and sutured with eight to ten interrupted sutures of 11/0 nylon (Ethicon, Somerville, New Jersey, USA) using microsurgical techniques. The total ischaemic time was kept to less than 30 min. Patency was verified under the stereomicroscope by the presence of pulsations proximal and distal to the interposed vein segment. A total of 28 rats recovered from anaesthesia; two animals died during the operation.

Animals were randomized to either NAC (14 rats) or control treatment (14). The respective groups received either 2 ml NAC solution (150 mg per kg bodyweight NAC in 0·9 per cent sodium chloride) or 2 ml saline once a day via oral force-feeding. NAC or control treatment was started 2 days before surgery and continued until death. Rats were killed 7 days (seven rats in the NAC group, seven in the control group) or 3 weeks (seven rats in the NAC group, seven in the control group) after vein graft surgery.

To investigate whether NAC limited smooth muscle cell proliferation, 5-bromo-2-deoxyuridine (BrdU) immunostaining was performed 7 days after venous grafting. It has been shown in a similar vein graft model that smooth muscle cell proliferation rates were highest 1 week after surgery[20]. BrdU is a synthetic nucleoside that is an analogue of thymidine; its immunohistochemistry is commonly used to detect proliferating cells in tissues. BrdU can be incorporated into the newly synthesized DNA of replicating cells during the S phase of the cell cycle, substituting for thymidine during DNA replication. Antibodies specific for BrdU are then used to detect the incorporated chemical, thus indicating cells that were actively replicating their DNA. BrdU (30 mg/kg) was administered into the peritoneum 18 and 12 h before euthanasia. Rats were anaesthetized, the chest and abdominal cavities were opened, and a catheter was inserted into the apex of the heart. Blood vessels were initially flushed with physiological saline solution and then perfusion fixed with 3·7 per cent formaldehyde in phosphate-buffered saline, pH 7·4, at physiological pressure (100 mmHg). Vein grafts were removed, fixed overnight in the same fixative, paraffin embedded and processed routinely.

Histomorphometric analysis

Cross-sections (4 μm) were taken for morphometric analysis at 200-μm intervals and mounted on glass slides for elastin staining (elastic van Gieson). The presence of VSMCs in media and neointima was determined by use of a monoclonal mouse anti-α-smooth muscle actin antibody that cross-reacts with rat (clone 1A4; Sigma-Aldrich, St Louis, Missouri, USA), and a secondary goat antimouse horseradish peroxidase-conjugated antibody (Sigma), followed by detection using diaminobenzidine as a substrate, and counterstaining with haematoxylin. Medial

thickness, neointimal area (area between the internal elastic lamina and endothelium) and percentage stenosis (area within the endothelium divided by the area within the internal elastic lamina) were determined on two midgraft sections, using a computer-assisted morphometry system (analySIS®; Soft Imaging System, Münster, Germany).

BrdU immunoreactivity was detected in vein graft segments by a specific monoclonal anti-BrdU antibody (1 in 500 dilution; Dako, Glostrup, Denmark). After DNA denaturation with 2 N hydrochloric acid and enzymatic pretreatment with pepsin, BrdU immunohistochemistry was performed with biotinylated goat antimouse immunoglobulin G (Dako) and the ABC-AP kit (Vector Laboratories, Burlingame, California, USA) with Fast Red (Vector Laboratories) and counterstaining with haematoxylin for quantification. A scale between 1 (few) and 4 (abundant) was used to obtain a semiquantitative index of the number of proliferating cells in the media and neointima of experimental vein grafts by an observer blinded to the specimen group.

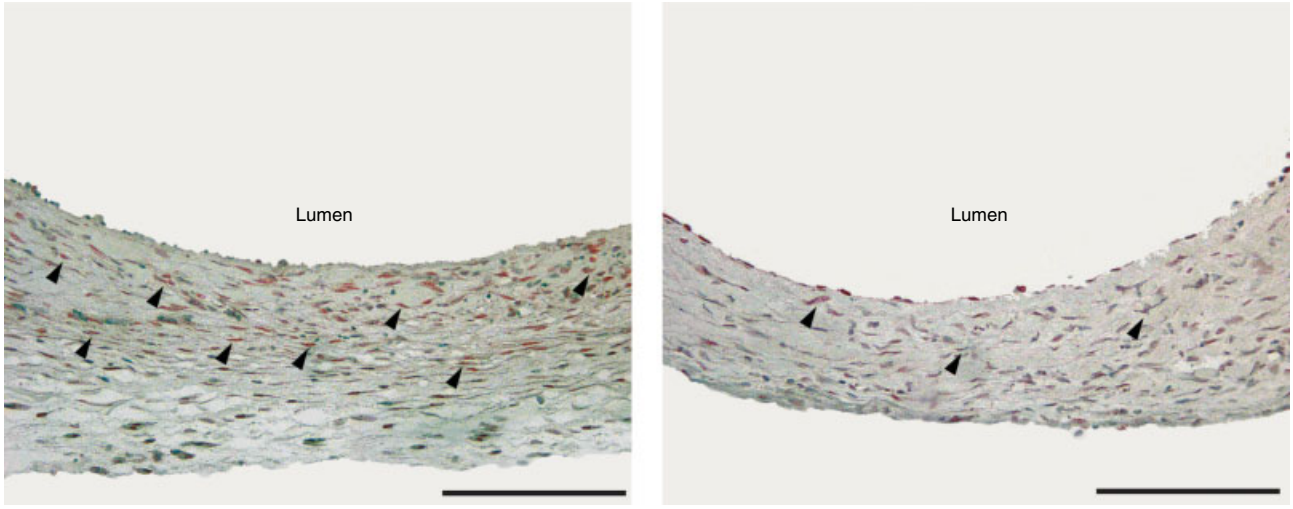
In vitro

Cell culture

Human venous smooth muscle cells (CRL-2482, iliac vein; ATCC, Manassas, Virginia, USA) were cultured in Kaighn's modification of Ham's F-12 (F-12K) nutrient medium (30–2004; ATCC), supplemented with 0·1 mg/ml heparin and 10 per cent fetal calf serum. Cells were grown under normoxic (21 per cent oxygen and 5 per cent carbon dioxide) conditions. Passages 4–6 were used for subsequent *in vitro* experiments.

Cytokine analysis

Cells were seeded at a density of 5×10^5 cells/ml in 24-well cell culture plates and synchronized for 24 h in F-12K medium supplemented with 2 per cent fetal calf serum under normoxic (21 per cent oxygen and 5 per cent carbon dioxide) conditions. Subsequently, quiescent VSMCs were placed in an incubator under hyperoxic (70 per cent oxygen and 5 per cent carbon dioxide) or normoxic (21 per cent oxygen and 5 per cent carbon dioxide) conditions. Values for the partial pressure of oxygen (PO_2) and of carbon dioxide (PCO_2) in the medium were measured in preliminary experiments with a blood gas analyser. The mean PO_2 was 714 and 173 mmHg under hyperoxic and normoxic conditions respectively. The PCO_2 and pH values of the culture medium were similar under normoxic and hyperoxic conditions. Medium and cells were collected after 12, 24, 48 and 72 h of incubation under either hyperoxic or normoxic conditions. Viability of the cells was determined by trypan blue staining. Conditioned medium was immediately analysed for cytokine concentration. The human inflammation Cytometric Bead Array kit (BD Biosciences, Franklin Lates, New Jersey, USA) was used to measure cytokine (Interleukin (IL) 1β, IL-6, IL-8, IL-10, tumour necrosis factor (TNF) α, IL-12p70) expression levels quantitatively in VSMC culture media. Some 50 μl conditioned medium was used and the assay was



a Control rats **b** *N*-acetylcysteine-treated rats

Fig. 1 Venous smooth muscle cell proliferation 7 days after surgery, as demonstrated by 5-bromo-2-deoxyuridine immunohistochemistry (arrowheads): it was significantly higher in **a** vein grafts of six control rats than in **b** seven rats that received *N*-acetylcysteine (150 mg per kg per day orally). Scale bars represent 100 μm

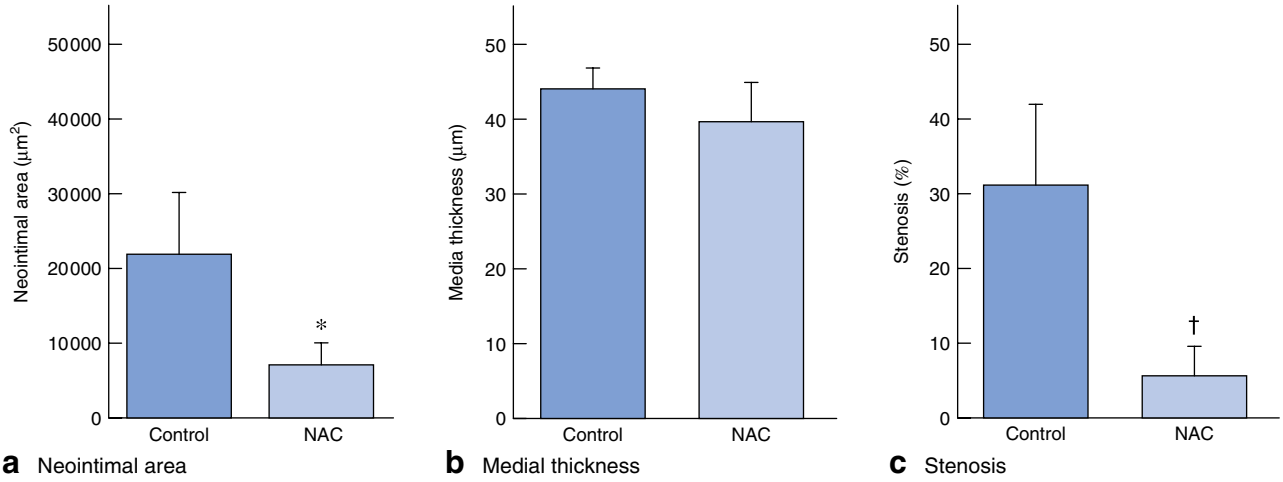


Fig. 2 **a** The neointimal area in vein grafts 3 weeks after surgery was significantly lower in six *N*-acetylcysteine (NAC)-treated (150 mg per kg per day orally) than in six control rats. **b** The medial thickness of grafted veins was not significantly different between NAC-treated and control groups. **c** The percentage graft stenosis was significantly lower in the NAC-treated group. Values are mean(s.e.m.). *$P < 0{\cdot}050$; †$P < 0{\cdot}010$ *versus* control (Mann–Whitney *U* test)

performed according to the manufacturer's instructions and analysed on the FACSCalibur™ flow cytometer (BD Biosciences).

Cell proliferation assay

VSMCs were seeded on glass coverslips (5000 cells/cm^2) in 24-well plates and synchronized for 24 h in F-12K medium, supplemented with 2 per cent fetal calf serum under normoxic conditions. Again, conditioned medium from VSMCs was collected after incubation for 12, 24, 48 and 72 h under either hyperoxic or normoxic conditions and transferred immediately to quiescent normoxic VSMCs, which were subsequently placed in an incubator under normoxic conditions for 48 h. After 48 h, cells were washed in phosphate-buffered saline, fixed in formaldehyde and permeabilized in Nonidet P-40 for 20 min. Non-specific binding sites were blocked with 2 per cent bovine serum albumin. VSMCs were incubated with a primary mouse anti-Ki-67 antibody that cross-reacts with human, and then a secondary goat antimouse antibody (both Dako). Finally, VSMCs were incubated with horseradish peroxidase-coupled streptavidin (Vector Laboratories), followed by detection using diaminobenzidine as a substrate, and counterstaining with haematoxylin. Ki-67 antigen is the prototypic cell cycle-related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G_1, S, G_2 and M phase). It is absent in resting (G_0) cells. Immunostaining with Ki-67 antibodies is routinely used as a marker of cell proliferation. For each experimental and control group, total nuclei and Ki-67-positive nuclei were counted by an observer blinded to the specimen group. The proliferation index was calculated as the amount of positive cells divided by total cell count.

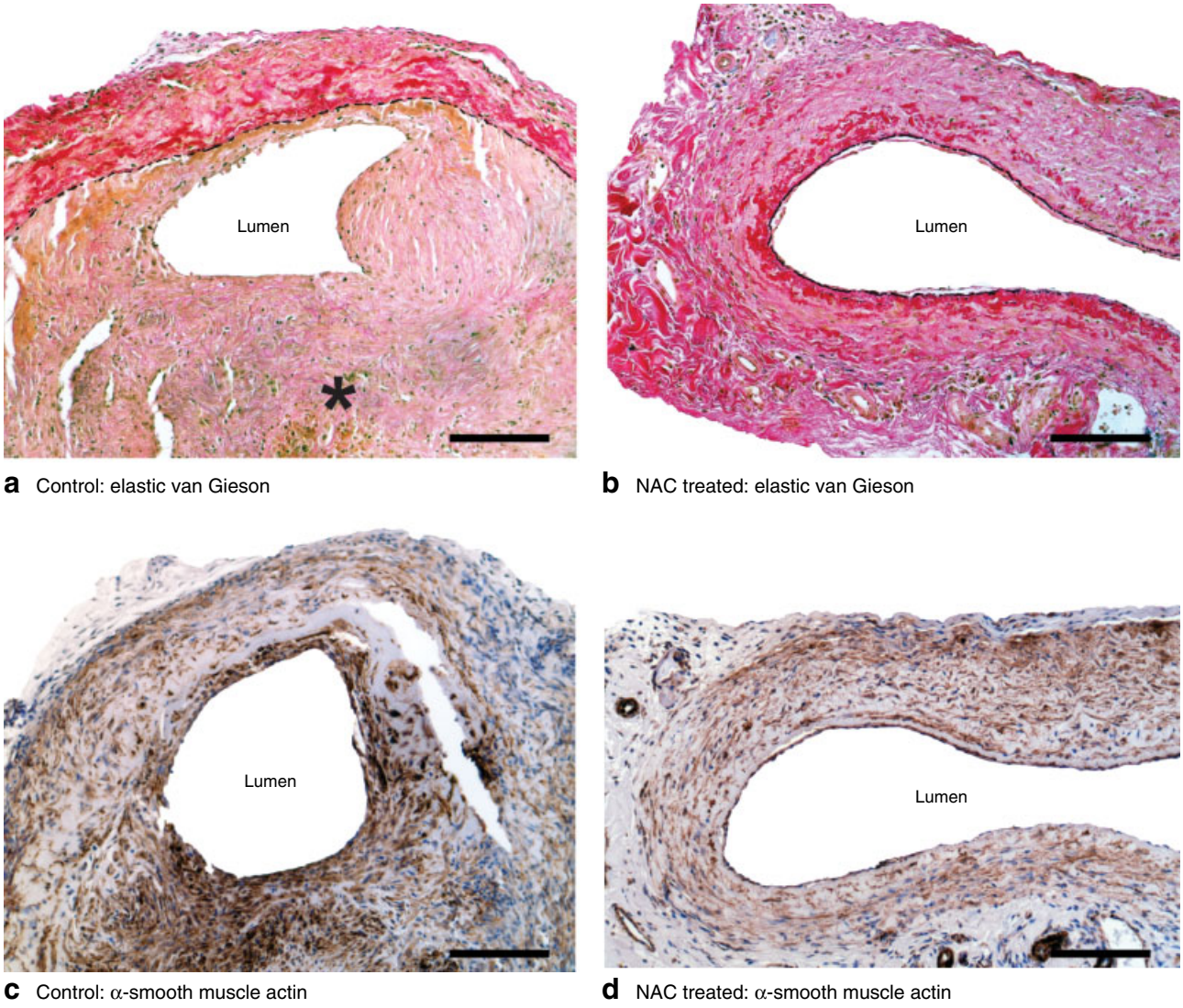
Effect of NAC on hyperoxia-induced cytokine production and VSMC proliferation

To examine the effect of NAC on hyperoxia-induced cytokine production in VSMCs, NAC (Sigma-Aldich Sweden, Stockholm, Sweden) was added to F-12K medium to a final concentration of 2 mmol/l. VSMCs were incubated with NAC-enriched medium under hyperoxic conditions (70 per cent oxygen and 5 per cent carbon dioxide). At 12, 24, 48 and 72 h after exposure to hyperoxia, 2 ml conditioned medium was removed and transferred immediately on to quiescent normoxic VSMCs, which were further incubated under normoxic conditions. After 48 h a proliferation assay was performed on these cells as described above. An additional 2 ml conditioned medium was used for cytokine quantification by means of a Cytometric Bead Array kit as described above.

Because NAC has been suggested to cause growth-arrest of smooth muscle cells[21], the direct effects of NAC on VSMCs stimulated by conditioned medium were also determined. Quiescent VSMCs were incubated with conditioned medium retrieved from hyperoxia-exposed VSMCs with the addition of 2 mmol/l NAC and a proliferation assay was performed as described.

Statistical analysis

Data were expressed as mean(s.e.m.). Differences between the experimental and control groups for cytokine analysis



a Control: elastic van Gieson **b** NAC treated: elastic van Gieson

c Control: α-smooth muscle actin **d** NAC treated: α-smooth muscle actin

Fig. 3 **a** Abundant neointima formation (*) was present in a vein graft segment from a control rat 3 weeks after surgery. **b** Treatment with *N*-acetylcysteine (NAC) prevented neointima formation, as shown by elastic van Gieson elastin staining. **c** Immunostaining with α-smooth muscle actin showed smooth muscle cells in the neointima of control vein grafts compared with a reduced number in **d** NAC-treated vein grafts. Scale bars represent 100 μm

and comparisons between groups for morphometric analyses were made using the Mann–Whitney *U* test. $P < 0{\cdot}050$ was considered significant.

Results

NAC treatment inhibited *in vivo* neointima formation in rat venous grafts

At 7 days after surgery, one vein graft in the control group was occluded by thrombosis and was excluded from analysis. None of the vein grafts in the NAC-treated group had thrombosed. Anti-BrdU staining was performed in all remaining vein graft segments (six in the control group and seven in the NAC group) (*Fig. 1*). Proliferation scores in the media and neointima of vein grafts were significantly lower in the NAC-treated group than in the control group (1·5(0·2) *versus* 3·1(0·2); $P = 0{\cdot}004$). Most cells in the vein segments were qualified as VSMCs, as determined by anti-α-smooth muscle actin staining.

Some 3 weeks after surgery, thrombosis was seen in one vein segment in the control and one from the treatment group. Morphological measurements were performed on the remaining vein graft segments (six each in the control

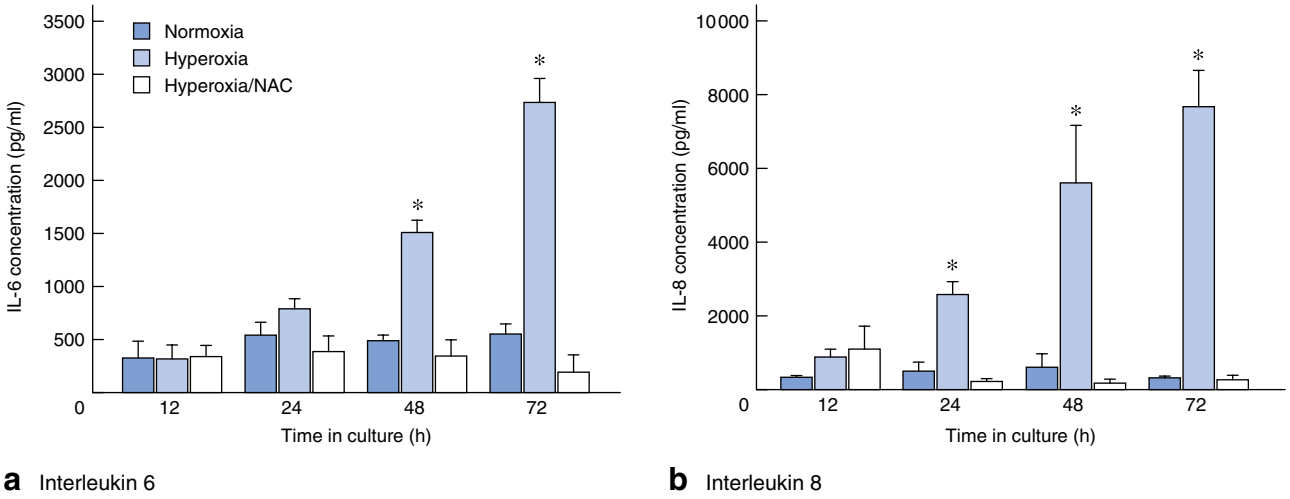


Fig. 4 Both **a** interleukin (IL) 6 and **b** IL-8 levels in the culture medium, as determined by Cytometric Bead Array assay, were significantly increased during exposure to 70 per cent oxygen (hyperoxia) compared with exposure to 21 per cent oxygen (normoxia). Concomitant administration of the antioxidant *N*-acetylcysteine (NAC) (hyperoxia/NAC) completely abolished hyperoxia-induced cytokine expression. Values are mean(s.e.m.) of four experiments. *$P < 0{\cdot}010$ *versus* control (Mann–Whitney *U* test)

and NAC groups). The neointimal area in the NAC-treated group was threefold lower than in the control group (*Figs 2a*, *3a* and *3b*). Furthermore, the luminal stenosis rate in grafted veins in the NAC-treated group was sixfold lower than in the control group (*Fig. 2c*). In contrast, medial thickness did not differ between treatment and control groups (*Fig. 2b*). Additionally, α-smooth muscle actin immunostaining demonstrated significantly fewer smooth muscle cells in the neointima of NAC-treated than in control vein grafts (*Figs 3c* and *3d*). Taken together, these results demonstrated that NAC inhibited neointima formation in experimental vein grafts, through reduction of VSMC proliferation.

NAC prevented hyperoxia-induced cytokine production and VSMC proliferation *in vitro*

Hyperoxia stimulated release of cytokines in VSMCs

Viability of cells was more than 95 per cent at 0 h, and more than 80 per cent at 72 h of hyperoxia or normoxia exposure as determined by trypan blue staining, suggesting that the chosen level of 70 per cent hyperoxia carried low cytotoxicity *in vitro*. VSMCs exposed to 70 per cent oxygen showed a time-dependent increase in the production of IL-6 and IL-8 compared with VSMCs incubated at 21 per cent oxygen (*Fig. 4*). In contrast, IL-10 levels in the medium were on average below the detection limit of the assay (3·3 pg/ml). No significant increase from baseline concentration of IL-1β, IL-12p70 or TNF-α was found in either hyperoxic or normoxic cells (*Table 1*).

Cytokine-rich medium from hyperoxic VSMCs induced proliferation of normoxic VSMCs

Quiescent VSMCs incubated under normoxic conditions were exposed for 48 h to medium retrieved from hyperoxia-exposed VSMCs. Direct cell counting revealed a significant increase in the number of Ki-67-positive VSMC nuclei incubated with conditioned medium compared with VSMCs incubated with control medium (*Fig. 5*). Incubation with conditioned medium obtained after 24, 48 and 72 h of hyperoxia induced a fourfold, sixfold and ninefold increase of Ki-67-positive cells respectively. Together, these data show that exposure of VSMCs to hyperoxia led to secretion of mitogenic cytokines IL-6 and IL-8, and proliferation of quiescent VSMCs.

NAC inhibited hyperoxia-induced cytokine release and VSMC proliferation

Subsequently, the therapeutic potential of NAC as an anti-inflammatory and antiproliferation agent was assessed *in vitro*. To determine the effects of NAC treatment on proinflammatory cytokine secretion from VSMCs, 2 mmol/l NAC was added to the culture medium and quiescent VSMCs were subsequently exposed to hyperoxia. Treatment with NAC prevented the increase in IL-6 and IL-8 levels after 24, 48 and 72 h of hyperoxia (*Fig. 4*), but did not affect the secretion of other measured cytokines (*Table 1*). Next, the mitogenic effects of conditioned medium retrieved from these VSMCs that received NAC throughout exposure to hyperoxia were determined. Proliferation rates of normoxic VSMCs

Table 1 Effects of hyperoxia and *N*-acetylcysteine on cytokine secretion by human venous smooth muscle cells

	Time after surgery (h)	IL-1β	IL-6	IL-8	IL-10	IL-12p70	TNF-α
Control	0	n.d.	30(12)	186(80)	n.d.	n.d.	n.d.
Control	12	35(10·6)	315(163)	323(49)	n.d.	3·1(1·2)	2·7(1·0)
O_2 − NAC	12	34(16·4)	309(134)	874(200)	n.d.	4·2(2·1)	2·4(0·9)
O_2 + NAC	12	31(9·6)	330(106)	1078(632)	n.d.	3·7(2·1)	2·2(1·0)
Control	24	30(9·5)	536(119)	485(234)	n.d.	5·0(2·8)	3·4(1·6)
O_2 − NAC	24	22(10·6)	780(100)	2560(351)†	n.d.	7·3(4·3)	2·4(1·1)
O_2 + NAC	24	31(8·7)	380(151)	209(67)	n.d.	5·5(2·7)	1·9(0·5)
Control	48	27(12·1)	485(49)	631(336)	n.d.	3·3(2·3)	2·3(0·8)
O_2 − NAC	48	26(12·5)	1503(120)†	5470(1509)*	n.d.	5·9(4·1)	2·5(0·9)
O_2 + NAC	48	26(11·3)	340(149)	210(90)	n.d.	3·8(2·4)	2·9(1·3)
Control	72	32(9·9)	546(94)	360(37)	n.d.	3·6(2·4)	2·3(0·2)
O_2 − NAC	72	34(11·3)	2729(223)†	7614(731)†	n.d.	9·3(3·9)	3·4(1·7)
O_2 + NAC	72	32(9·3)	187(164)	315(121)	n.d.	6·5(2·9)	2·1(0·8)

Values are mean(s.e.m.) of four experiments and all values are expressed in pg/ml. IL, interleukin; TNF, tumour necrosis factor; control, exposure to 21 per cent oxygen; n.d., not detectable; O_2 − NAC, exposure to 70 per cent oxygen without *N*-acetylcysteine (NAC); O_2 + NAC, exposure to 70 per cent oxygen with NAC. *$P < 0·050$, †$P < 0·010$ *versus* control (Mann–Whitney *U* test).

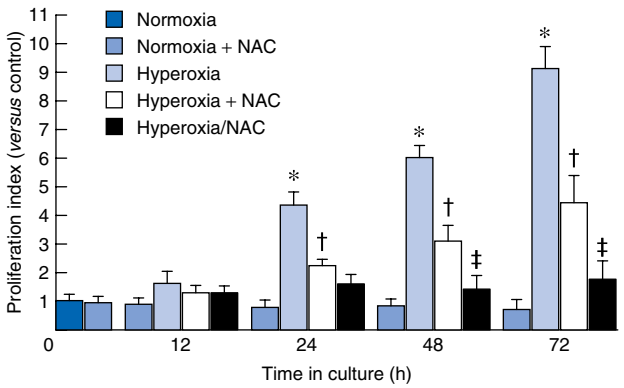


Fig. 5 Proliferation indices in normoxic venous smooth muscle cells (VSMCs) after 48 h with conditioned medium retrieved from VSMCs after 24–72 h of hyperoxia induced a significant increase in the number of Ki-67 positive cells (*$P < 0·010$ *versus* incubation with control medium). Administration of *N*-acetylcysteine (NAC) to quiescent VSMCs reduced the proliferative effect of medium from hyperoxic VSMCs (†$P < 0·050$ *versus* medium from hyperoxic VSMCs). The proliferative response of quiescent VSMCs was completely abolished by conditioned medium from hyperoxic NAC-treated VSMCs (‡$P < 0·050$ *versus* medium from hyperoxic VSMCs not treated with NAC). Values are mean(s.e.m.) of four experiments. Differences were tested with Mann–Whitney *U* test

after the 48-h incubation with conditioned medium from hyperoxic NAC-treated VSMCs were significantly reduced to quiescent normoxic levels compared with incubation with medium from hyperoxic VSMCs that had not been treated with NAC (*Fig. 5*). These data indicated that medium retrieved from hyperoxia-exposed and NAC-treated VSMCs lost its mitogenic potential.

NAC treatment of quiescent normoxic VSMCs reduced the proliferative response of these cells to conditioned medium from hyperoxia-exposed VSMCs, supporting a direct inhibitory effect of NAC on VSMC proliferation. However, this direct antiproliferative effect of NAC on normoxic VSMCs was significantly smaller than the inhibitory effect of NAC treatment of hyperoxic VSMCs on subsequent normoxic VSMC proliferation (*Fig. 5*).

Discussion

The present study examined the potential of NAC to prevent vein graft stenosis in a rat model and showed that NAC attenuated neointima formation and vein graft stenosis by reducing VSMC proliferation.

NAC has been shown to be of benefit in many clinical conditions, including sepsis, respiratory failure, hepatic failure and prevention of radiographic contrast nephropathy[22]. Furthermore, NAC may reduce arterial stenosis in experimental models of balloon-induced arterial injury[23,24].

When a vein segment is implanted into the arterial circulation, it becomes exposed to oxygen-rich blood, which might lead to oxidative stress in the venous tissue. The antioxidant NAC is a thiol compound which, by providing sulfhydryl groups, can act both as a precursor of reduced glutathione and as a direct reactive oxygen species scavenger. Hence, NAC modulates the activity of redox-sensitive cell-signalling and transcription pathways that play a role in inflammatory responses, cell growth and arrest, angiogenesis and apoptosis[25].

In the present study, NAC prevented a hyperoxia-induced increase in secretion of the mitogenic cytokines

IL-6 and IL-8 by VSMCs *in vitro*. The potential effects of hyperoxia on the vein wall were investigated in smooth muscle cells, not in endothelial cells, as vein grafts are rapidly denuded of their endothelium by surgical manipulation and haemodynamic stress. Exposure of isolated human VSMCs to 70 per cent oxygen induced a marked increase in IL-6 and IL-8 secretion, whereas the levels of IL-1β, TNF-α and IL-10 remained unaffected. Induction of IL-6 and IL-8 secretion under hyperoxic conditions has previously been shown in macrophages[26,27]. The only study so far to have examined the effects of hyperoxia on vascular wall cells found an increase in IL-8 mRNA levels in human umbilical vein endothelial cells[28]. Reports on the expression of other inflammatory cytokines, including IL-1β, are conflicting, as both increased and decreased expression have been reported in response to hyperoxia[26,28,29].

The proinflammatory cytokines IL-6 and IL-8 have been shown to exhibit mitogenic properties in smooth muscle cells[30–32]. Indeed, in the present study, exposure of normoxic quiescent VSMCs to IL-6 and IL-8-rich culture medium retrieved from hyperoxic VSMCs induced a proliferative response. NAC abolished this proliferative response. In contrast to the cytokine-mediated proliferative effect of hyperoxia, direct exposure of VSMCs to hyperoxia did not lead to proliferation (data not shown). Congruent with these findings, previous studies demonstrated that *in vitro* hyperoxia induces growth arrest in the S phase of the cell cycle[33]. The effects of hyperoxia on smooth muscle cell proliferation *in vivo* may relate to the position of the smooth muscle cell within the vascular wall. Arterial P_{O_2} decreases from 90 to 35 mmHg within a 1-mm distance in the vessel wall as measured from the lumen[34]. Although high luminal oxygen concentration might lead to growth arrest in the first cell layers, it may be speculated that paracrine stimulation by high levels of IL-6 and IL-8 secreted by the hyperoxic cells might induce smooth muscle cell proliferation in the medial cell layers.

The effect of NAC on hyperoxia-induced cytokine production and VSMC proliferation *in vitro* may be explained by its antioxidant potential, by scavenging reactive oxygen species and restoring cellular redox status. Alternatively, NAC may reduce the DNA-binding activity of the nuclear transcription factor nuclear factor κB, thereby decreasing transcription of *IL-6* and *IL-8* genes[35,36]. In addition to inhibiting the proliferative response to conditioned medium retrieved from NAC-treated hyperoxic VSMCs, NAC administration to quiescent VSMCs also limited their proliferative response to incubation with cytokine-rich medium from hyperoxic VSMCs. The latter finding supports a direct antiproliferative effect of NAC on VSMCs[37].

Although NAC prevented hyperoxia-induced cytokine production and VSMC proliferation *in vitro*, these data cannot be directly extrapolated to the *in vivo* situation. Partial oxygen pressures commonly used in cell culture conditions to assess the effect of hyperoxia do not reflect physiological pressures. Normoxia for cells is an adjustable variable that is dependent on the specific localization of the cell in organs and functional status of the specific tissue. P_{O_2} in mammalian organs ranges from 90 to below 3 mmHg, with arterial P_{O_2} of about 100 mmHg or 14 per cent oxygen[38]. Intracellular oxygen concentration must be maintained within narrow normoxic limits to circumvent metabolic demise from hypoxia or oxidative damage from hyperoxia[39]. Although current work in this field is almost exclusively focused on the study of hypoxia, the sensing of ambient oxygen is not limited to hypoxia. Hyperoxia affects genes associated with cell–cell communication and cell growth[40]. It has been demonstrated that exposure to a higher P_{O_2}, relative to which cells are adjusted, triggers phenotypic changes in cardiac fibroblasts associated with tissue remodelling following cardiac ischaemia–reperfusion[17,41]. In line with this, the present findings support the contention that environmental changes in oxygen may be a cue that VSMCs respond to. The concept of hyperoxia in the complex pathophysiology of neointima formation is a potential advance for further research.

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R. de Graaf and A. Tintu contributed equally to the manuscript.

References

1 Motwani JG, Topol EJ. Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. *Circulation* 1998; **97**: 916–931.
2 Gaudino M, Cellini C, Pragliola C, Trani C, Burzotta F, Schiavoni G *et al*. Arterial *versus* venous bypass grafts in patients with in-stent restenosis. *Circulation* 2005; **112**(Suppl 9): 1265–1269.
3 Cox JL, Chiasson DA, Gotlieb AI. Stranger in a strange land: the pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences between veins and arteries. *Prog Cardiovasc Dis* 1991; **34**: 45–68.

4 Deng Z, Wang Y, Jiang K, Liu X, Wu W, Gao S *et al*. Molecular cloning and characterization of a novel dehydrin gene from *Ginkgo biloba*. *Biosci Rep* 2006; **26**: 203–215.
5 Yang Z, Oemar BS, Carrel T, Kipfer B, Julmy F, Luscher TF. Different proliferative properties of smooth muscle cells of human arterial and venous bypass vessels: role of PDGF receptors, mitogen-activated protein kinase, and cyclin-dependent kinase inhibitors. *Circulation* 1998; **97**: 181–187.
6 Heistad DD. Oxidative stress and vascular disease: 2005 Duff lecture. *Arterioscler Thromb Vasc Biol* 2006; **26**: 689–695.
7 Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part II: animal and human studies. *Circulation* 2003; **108**: 2034–2040.
8 Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol* 2005; **25**: 29–38.
9 Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 2000; **86**: 494–501.
10 Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000; **87**: 840–844.
11 Shi Y, Patel S, Davenpeck KL, Niculescu R, Rodriguez E, Magno MG *et al*. Oxidative stress and lipid retention in vascular grafts: comparison between venous and arterial conduits. *Circulation* 2001; **103**: 2408–2413.
12 Hishikawa K, Luscher TF. Pulsatile stretch stimulates superoxide production in human aortic endothelial cells. *Circulation* 1997; **96**: 3610–3616.
13 O'Brien JE Jr, Ormont ML, Shi Y, Wang D, Zalewski A, Mannion JD. Early injury to the media after saphenous vein grafting. *Ann Thorac Surg* 1998; **65**: 1273–1278.
14 Nunes GL, Robinson K, Kalynych A, King SB III, Sgoutas DS, Berk BC. Vitamins C and E inhibit O_2^- production in the pig coronary artery. *Circulation* 1997; **96**: 3593–3601.
15 Jones R, Zapol WM, Reid L. Pulmonary artery remodeling and pulmonary hypertension after exposure to hyperoxia for 7 days. A morphometric and hemodynamic study. *Am J Pathol* 1984; **117**: 273–285.
16 Coflesky JT, Jones RC, Reid LM, Evans JN. Mechanical properties and structure of isolated pulmonary arteries remodeled by chronic hyperoxia. *Am Rev Respir Dis* 1987; **136**: 388–394.
17 Roy S, Khanna S, Wallace WA, Lappalainen J, Rink C, Cardounel AJ *et al*. Characterization of perceived hyperoxia in isolated primary cardiac fibroblasts and in the reoxygenated heart. *J Biol Chem* 2003; **278**: 47 129–47 135.
18 Hoch JR, Stark VK, Hullett DA, Turnipseed WD. Vein graft intimal hyperplasia: leukocytes and cytokine gene expression. *Surgery* 1994; **116**: 463–470.
19 Hirsch GM, Karnovsky MJ. Inhibition of vein graft intimal proliferative lesions in the rat by heparin. *Am J Pathol* 1991; **139**: 581–587.
20 Dilley RJ, McGeachie JK, Tennant M. The role of cell proliferation and migration in the development of a neo-intimal layer in veins grafted into arteries, in rats. *Cell Tissue Res* 1992; **269**: 281–287.
21 Lee JS, Kypreos KE, Sonenshein GE. Synchronization of cultured vascular smooth muscle cells following reversal of quiescence induced by treatment with the antioxidant *N*-acetylcysteine. *Exp Cell Res* 1998; **239**: 447–453.
22 Atkinson MC. The use of *N*-acetylcysteine in intensive care. *Crit Care Resusc* 2002; **4**: 21–27.
23 Hayashi K, Takahata H, Kitagawa N, Kitange G, Kaminogo M, Shibata S. *N*-acetylcysteine inhibited nuclear factor-kappaB expression and the intimal hyperplasia in rat carotid arterial injury. *Neurol Res* 2001; **23**: 731–738.
24 Ghigliotti G, Mereto E, Eisenberg PR, Martelli A, Orsi P, Sini D *et al*. *N*-acetyl-cysteine reduces neointimal thickening and procoagulant activity after balloon-induced injury in abdominal aortae of New Zealand white rabbits. *Thromb Haemost* 2001; **85**: 724–729.
25 Sadowska AM, Manuel-Y-Keenoy B, De Backer WA. Antioxidant and anti-inflammatory efficacy of NAC in the treatment of COPD: discordant *in vitro* and *in vivo* dose-effects: a review. *Pulm Pharmacol Ther* 2007; **20**: 9–22.
26 Desmarquest P, Chadelat K, Corroyer S, Cazals V, Clement A. Effect of hyperoxia on human macrophage cytokine response. *Respir Med* 1998; **92**: 951–960.
27 Hjort MR, Brenyo AJ, Finkelstein JN, Frampton MW, LoMonaco MB, Stewart JC *et al*. Alveolar epithelial cell–macrophage interactions affect oxygen-stimulated interleukin-8 release. *Inflammation* 2003; **7**: 137–145.
28 Park MS, Wallace HM. Hyperoxia influences mRNA expression of cytokines in cultured human umbilical vein endothelial cells. *Yonsei Med J* 1998; **39**: 1–12.
29 Bhandari V, Elias JA. Cytokines in tolerance to hyperoxia-induced injury in the developing and adult lung. *Free Radic Biol Med* 2006; **41**: 4–18.
30 Christiansen JF, Hartwig D, Bechtel JF, Klüter H, Sievers H, Schönbeck U *et al*. Diseased vein grafts express elevated inflammatory cytokine levels compared with atherosclerotic coronary arteries. *Ann Thorac Surg* 2004; **77**: 1575–1579.
31 Yue TL, Wang X, Sung CP, Olson B, McKenna PJ, Gu JL *et al*. Interleukin-8. A mitogen and chemoattractant for vascular smooth muscle cells. *Circ Res* 1994; **75**: 1–7.
32 Ross R. Atherosclerosis – an inflammatory disease. *N Engl J Med* 1999; **340**: 115–126.
33 Shenberger JS, Dixon PS. Oxygen induces S-phase growth arrest and increases p53 and p21(WAF1/CIP1) expression in human bronchial smooth-muscle cells. *Am J Respir Cell Mol Biol* 1999; **21**: 395–402.
34 Santilli SM, Wernsing SE, Lee ES. The effect of supplemental oxygen on the transarterial wall oxygen gradients at a prosthetic vascular graft to artery anastomosis in the rabbit. *Ann Vasc Surg* 2001; **15**: 435–442.
35 Lappas M, Permezel M, Rice GE. *N*-Acetyl-cysteine inhibits phospholipid metabolism, proinflammatory cytokine release, protease activity, and nuclear factor-kappaB deoxyribonucleic

acid-binding activity in human fetal membranes *in vitro*. *J Clin Endocrinol Metab* 2003; **88**: 1723–1729.
36 Paterson RL, Galley HF, Webster NR. The effect of *N*-acetylcysteine on nuclear factor-kappa B activation, interleukin-6, interleukin-8, and intercellular adhesion molecule-1 expression in patients with sepsis. *Crit Care Med* 2003; **31**: 2574–2578.
37 Li J, Tu HJ, Dai G, Dai YC, Wu Q, Shi QZ *et al*. *N*-acetyl cysteine inhibits human signet ring cell gastric cancer cell line (SJ-89) cell growth by inducing apoptosis and DNA synthesis arrest. *Eur J Gastroenterol Hepatol* 2007; **19**: 769–774.
38 Porwol T, Ehleben W, Brand V, Acker H. Tissue oxygen sensor function of NADPH oxidase isoforms, an unusual cytochrome aa3 and reactive oxygen species. *Respir Physiol* 2001; **128**: 331–348.
39 Semenza GL. HIF-1, O_2, and the 3 PHDs: how animal cells signal hypoxia to the nucleus. *Cell* 2001; **107**: 1–3.
40 Sen CK, Khanna S, Roy S. Perceived hyperoxia: oxygen-induced remodeling of the reoxygenated heart. *Cardiovasc Res* 2006; **71**: 280–288.
41 Roy S, Khanna S, Bickerstaff AA, Subramanian SV, Atalay M, Bierl M *et al*. Oxygen sensing by primary cardiac fibroblasts: a key role of p21(Waf1/Cip1/Sdi1). *Circ Res* 2003; **92**: 264–271.

25 years ago

Elastic band ligation of haemorrhoids: a new applicator

Description of the Pyser suction applicator for haemorrhoidal banding.

"Elastic band ligation of internal haemorrhoids is a well recognized procedure and can be used in the Out Patient Department. One disadvantage, however, is the need for two hands to hold the banding device and forceps and an assistant to hold the proctoscope. The device [Pyser haemorrhoid ligator] described overcomes this problem... The applicator was easy to use and the technique was quickly learned. None of the in-patients treated complained of severe postoperative pain and indeed most had no pain.

Four required a single dose of a minor oral analgesic. They were all discharged on the day following surgery and none returned with complications. The use of the applicator in the Out Patient Department proved easy and quick. All the patients had their haemorrhoids dealt with at one attendance and none returned with pain or bleeding following the application."

Schofield PF, Cunliffe WJ, Hulton N. Elastic band ligation of haemorrhoids: a new applicator. *Br J Surg* 1984; **71**: 212. (DOI: 10.1002/bjs.1800710316)

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