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# Vitamins C and E attenuate plasminogen activator inhibitor-1 (PAI-1) expression in a hypercholesterolemic porcine model of angioplasty

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## Abstract

**Background:** The plasminogen activator inhibitor-1 (PAI-1), which modulates fibrinolysis and cell migration, may influence proteolysis and neointimal formation in the arterial wall contributing to restenosis after vascular injury. Antioxidants have been proposed as inhibiting multiple proatherogenic events. We explore the effect of vitamins C and E on PAI-1 expression in an experimental model of angioplasty in hypercholesterolemic pigs. **Methods and results:** A total of 44 Yucatan minipigs were divided into three diet groups: a normal-cholesterol (NC), a high-cholesterol (HC), and a high-cholesterol plus vitamins C+E (HCV) group. Balloon injury was induced in the right internal iliac artery 4 weeks after initiation of either dietary regimen, and plasma and tissue samples were taken at different time periods to measure PAI-1 activity and vascular inhibitor expression. The cholesterol-rich diet induced an increased in vascular PAI-1 expression in the intima, media and adventitia which was markedly reduced in the HCV group. After injury, severe structural changes were observed in NC and HC animals associated with increased systemic PAI-1 activity ( $P < 0.001$ ) and local PAI-1 expression being more intense in HC group. Vitamins C and E significantly reduced plasma PAI-1 activity ( $P = 0.018$ ) and attenuated the inhibitor expression as compared with HC. **Conclusions:** This experimental study in a porcine model of hypercholesterolemia demonstrates that vitamins C and E reduce local and systemic PAI-1 induced after angioplasty as well as the hypercholesterolemia-induced vascular PAI-1. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Atherosclerosis; Cholesterol; Angioplasty; Hemostasis; Restenosis

## 1. Introduction

Percutaneous angioplasty is an established treatment for atherothrombosis. Despite a primary success rate, angioplasty induces early reocclusion in 3–13% and late restenosis in more than 30% of patients treated. The mechanisms promoting these lesions are not completely understood and different pharmacological strategies have not proved effective in preventing these complications although the introduction of intracoronary stents has reduced their incidence in recent years [1–3].

An injury-related healing response mediated by smooth muscle cell (SMC) proliferation and neointima formation, leading to luminal narrowing, has been considered an essential factor in the development of late restenosis. Recent data also indicate that vascular remodeling is an important pathophysiological mechanism for restenosis [4–7].

The plasminogen activator/plasmin system has been implicated in several cardiovascular disorders, including wound healing after vascular reconstruction as suggested from data obtained in PAI-1 knockout mice [8,9]. Recent observations indicate that components of this system may influence the process of restenosis by either promoting or inhibiting the development of the neointima [10–12]. PAI-

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1, the primary physiological inhibitor of plasminogen activators, is an important determinant of fibrinolysis at sites of vascular injury and has been implicated in the pathogenesis of thrombosis at the arterial site [13,14]. Clinical studies have suggested a relationship between PAI-1 in plasma and cardiovascular risk [15–17], and it has also been reported that the levels of PAI-1 mRNA are significantly elevated in atherosclerotic vessels and correlate with the severity of atherosclerotic lesions [14,18–20].

Antioxidant vitamins C and E have been evaluated through clinical studies for their effect on the prevention and treatment of atherosclerotic disease, although there is controversy surrounding the results [21]. At the experimental level, treatment with antioxidants has been shown to reduce intimal lesions after injury in hypercholesterolemic animals [22,23] and may contribute to plaque stabilization [24], but its possible effect on the regulation of fibrinolysis has not been properly assessed.

We therefore tried to analyze the local and systemic changes of PAI-1 in an experimental model of angioplasty induced in hypercholesterolemic pigs, on the basis they would likely predispose to thrombosis and possibly restenosis [25]. We tested further the hypothesis that vitamins C and E would favorably alter vascular PAI-1 expression induced by hypercholesterolemia and mechanical injury in this experimental model.

## 2. Methods

### 2.1. Animals

A total of 44 Yucatan minipigs (4 months old, mean weight  $33.29 \pm 1.69$  kg), procured from our breeding center, were maintained in the animal facilities of CIFA (GLP accredited center at the University of Navarra, Spain) in accordance with the *Guide for the Care and Use of Laboratory Animals* published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 2.2. Hypercholesterolemic model

Animals were divided into three groups and fed on different dietary formulations for 4 weeks: a normal-cholesterol (NC) control group ( $n=16$ ), fed standard porcine chow (Porcisanthers, Sanders, Spain); a high-cholesterol (HC) group ( $n=16$ ) fed with a diet containing 24.5% animal lard, 4% cholesterol (Roig Farma, Spain) and 1.5% bile extract (Roig Farma, Spain); and a high-cholesterol plus vitamins C and E (HCV) group ( $n=12$ ) fed in the same way as HC group but supplemented during the final week with 1 g vitamin C and 1000 IU vitamin E (Roig Farma, Spain) per animal per day. At this time, animals were subjected to catheterization and allowed to recover by staying on each diet until sacrifice. Vascular injury was induced in the right internal iliac arteries,

leaving left internal iliac arteries as control. A total of four animals from NC and HC groups were sacrificed at days 3, 7, 14 and 28 after the first intervention with a lethal dose of 65 mg/kg pentobarbital (Sigma, USA) followed by a bolus of KCl. Experiments were not performed at day 3 in the HCV group (total number included=12) based on the unlikely short-term effect of the treatment.

### 2.3. Arterial injury

Each animal was fasted overnight before the day of the study but allowed ad libitum access to tap water. Animals were sedated, intubated and ventilated through an endotracheal tube. Anesthetic induction was completed by i.v. injection of 2 mg/kg metomidate (Esteve Veterinaria, Spain); 0.2 mg/l pancuronium bromide (Organon, The Netherlands) and 0.05 mg fentanyl (Roche, Switzerland) were given to maintain a constant level of anesthesia. The right internal jugular vein and carotid artery were exposed by cutdown and cannulated with a 7F venous sheath and 9F arterial sheath, respectively.

After iliac angiography, endothelial injury was performed by three 30-s inflations of a balloon catheter at 8 atm, separated by 30-s intervals, in the medial portion of the internal iliac artery. The balloon diameter was chosen in order to achieve a balloon/artery diameter ratio higher than 1.2.

Arterial blood samples were drawn before and after injury for analytical measurements and vascular tissues were obtained for histological purposes.

### 2.4. Tissue processing

The iliac arterial tree was dissected, perfused with 4% paraformaldehyde at 4°C for 10 min and left in 500 ml of 4% paraformaldehyde at 4°C for 4 h. Arteries were cut to 1-cm-long pieces and paraffin embedded (up to eight pieces per block). Serial sections were cut (5  $\mu$ m) for in situ hybridization (ISH) and immunohistochemistry, and mounted on microscope slides (ProbeOn Plus, Fisher-Scientific, USA). Adjacent sections were stained with hematoxylin-eosin or elastin (van Giesson) for morphometric studies. Sections with maximal lesion were chosen for analysis.

### 2.5. Morphometric analysis

Stained arterial sections were examined with a microscope (Nikon Optiphot-2, Japan). Images were acquired with a video camera (Sony DX-151 AP, Japan) attached to a computer, and analyzed with Optimas 5.2 (Media Cybernetics, USA) software. The following parameters were measured: lumen area circumscribed by the intima/neointima lumina border; intima/neointima area between the lumen and the internal elastic lamina; media area between internal and external elastic lamina; cell density,

number of cells in 0.01 mm<sup>2</sup> of the media; percentage stenosis, defined as  $100 \times (1 - \text{lumen area} / \text{lumen area} + \text{neointima area})$ .

## 2.6. *In situ* hybridization (ISH)

A 434-bp fragment (nucleotides 217–651) derived from human PAI-1 cDNA [26] was amplified by PCR and subcloned in the PGEM 3Z vector (Promega, USA). Sense and antisense probes were prepared by linearizing the constructs with appropriate restriction enzymes and transcribed with either T7 and SP6 RNA polymerase (Roche, Switzerland) in the presence of DIG-11-UTP (Roche, Switzerland). ISH was performed according to Dijkman et al. [27], with some modifications. Arterial tissue sections from animals were deparaffinized in xylene, rehydrated in ethanol, postfixated with paraformaldehyde for 10 min at 4°C and treated with proteinase K 10 µg/ml (30 min at 37°C) (Roche, Switzerland). Samples were acetylated, washed and air dried. The cRNA probes were prepared at the concentration of 2.5 ng/µl (in 50% formamide, SSC 2×/dextran 10%, EDTA 5 mM, 10 mM DTT, 0.01% SDS, 1× Denhardt's solution and 250 µg/ml salmon sperm). Sections were covered with 20 µl of probe solution, sealed with coverslips and left hybridizing overnight, at 43°C in a humidified chamber. Samples were washed with 50% formamide/2× SSC at 42°C, 1× SSC at RT and 0.5× SSC before immunological detection with anti-DIG antibody and X-Phosphate/NBT as substrate. The reaction was stopped 2 h after the addition of substrate solution.

## 2.7. Immunohistochemistry

Sections were deparaffinized, rehydrated, and treated with 3% H<sub>2</sub>O<sub>2</sub> solution for 10 min to inhibit endogenous peroxidase. Slides were covered with a solution containing 0.1% w/v trypsin (T-4799, Sigma, USA), 0.1% w/v CaCl<sub>2</sub>, 0.05 mol/l Tris-HCl, pH 7.6, for 10 min at 37°C [28]. Non-specific binding was blocked by incubation with normal rabbit serum diluted 1:20 in TBS for 30 min and sections were incubated overnight at 4°C with 100 µg/ml anti-PAI-1 (#3785, American Diagnostica, USA) which recognizes inhibitor both free and complexed to plasminogen activators. After washing with TBS, sections were incubated for 1 h in 1:200 biotinylated rabbit anti-mouse Fc (Dako, Denmark). Slides were treated for 1 h with the avidin-biotin-peroxidase complex (ABC, Dako, Denmark) diluted 1:100. Peroxidase activity was detected with 3-3'-diaminobenzidine hydrochloride (Sigma, USA) and H<sub>2</sub>O<sub>2</sub>, with nickel enhancement, the time of development being the same for all samples [29]. Slides were counterstained with Harris' hematoxylin, dehydrated and mounted in DPX (BDH, UK). Negative controls (without primary antibody) yielded no immunohistochemical reaction.

## 2.7.1. Semi-quantitative assessments

A visual grading scale for the assessment of the intensity of ISH and immunohistochemistry staining was performed in batches of 12 slides and compared with internal control sections (scored 0 and 3). Sections were characterized by two observers independently, who were blinded to the characteristics of the samples. The mean scoring system of PAI-1 staining in relation to the number of cells/layer was established as follows: 0, no staining; 1, positive staining <25%; 2, positive staining >25%<75%; 3, positive staining >75%.

## 2.8. PAI-1 activity, cholesterol and vitamin E measurements

Blood samples were collected in 0.13 mol/l sodium citrate (9/1, v/v) kept on ice for no longer than 2 h and the platelet-poor plasma, obtained by centrifugation at 2000×g for 20 min at 4°C, was stored at -70°C until used.

PAI-1 activity was measured by an amidolytic assay [30], using a commercially available kit (Coatest-PAI, Chromogenix, Sweden). Assay specificity was assessed by using serial dilutions of human t-PA (0–100 IU/ml).

Serum cholesterol levels were determined by the standard enzymatic assay of Siedel et al. [31], using a fully automated clinical analyzer (Hitachi-717, Roche, Switzerland).

Vitamin E was measured in plasma using a reverse-phase high-performance liquid chromatography (HPLC) method [32].

## 2.9. Statistical analysis

All data were expressed as mean±S.E.M. Comparisons between different time periods within the same diet were done by the Wilcoxon signed rank test. Differences between groups were calculated by analysis of variance (ANOVA) followed by Tukey posthoc test or by Kruskal-Wallis, for parametric and non-parametric conditions, respectively. Statistical significance was accepted at the 95% confidence level ( $P < 0.05$ ). The correlation coefficient between variables was calculated using Spearman's correlation test for independent data. Histomorphological differences in response to vascular injury among groups were assessed using  $\chi^2$ -test. Analysis was performed by using the computer program SPSS for Windows (SPSS, USA).

## 3. Results

### 3.1. Hypercholesterolemia-induced vascular changes

#### 3.1.1. Morphometric analysis

Hypercholesterolemic diet substantially increased plasma cholesterol levels ( $1.5 \pm 0.1$  in NC vs.  $4.6 \pm 0.5$  mM in



HC,  $P<0.01$ ) after 4 weeks of diet. Hematoxylin-eosin staining of control arterial sections revealed intact endothelial structure in all groups. The main morphological changes in the HC group as compared with NC group consisted of a significant increase in the percentage stenosis ( $14.7\pm 3.3$  vs.  $5.7\pm 0.3\%$ ,  $P<0.05$ ) and intimal area ( $0.09$  vs.  $0.05$  mm<sup>2</sup>,  $P<0.01$ ), as well as a significant reduction in media cell density ( $38.8\pm 7.9$  vs.  $47.3\pm 3.9$  10<sup>2</sup> cells/mm<sup>2</sup>,  $P<0.05$ ), without differences between groups for other morphologic parameters analyzed.

### 3.1.2. PAI-1 expression

An intense and extensive vascular PAI-1 positive signal in the media area was observed both by immunohistochemistry (Fig. 1A,B) and ISH (Fig. 1D,E) in the HC group as compared with NC after 8 weeks of cholesterol rich diet. Results regarding semiquantitative assessment of immunohistochemistry and ISH in both groups are shown in Fig. 2A,B. Increased PAI-1 expression was observed in media layer of HC with respect to NC ( $P<0.01$ ). Furthermore, the number of arteries that exhibited increased

vascular PAI-1 was higher in HC than in NC group (25 vs. 6%).

The plasma PAI-1 activity, which was similar in both groups in baseline conditions, did not experience further increase despite the cholesterol feeding ( $28.1\pm 0.7$  vs.  $28.6\pm 0.2$  AU/ml). Cholesterol rich diet did not induce significant changes in plasma vitamin E levels between HC and NC groups ( $1.7\pm 0.2$  vs.  $2.0\pm 0.4$  μM, respectively).

### 3.2. Mechanical-induced vascular injury

#### 3.2.1. Morphometric analysis

Angioplasty induced severe structural changes in the vascular wall of both groups. A significant increase in the intimal area and percentage stenosis ( $P<0.05$ ) was present after vascular lesion as compared with non-injured arteries in NC animals. Changes were evident at 7 days reaching significance ( $P<0.05$ ) 28 days after injury (Fig. 3A,B), but occurred earlier in the HC than in the NC group. A significant ( $P<0.05$ ) time-dependent increase in intimal area, percentage stenosis and intima/media ratio was already observed 7 days after angioplasty and persisted

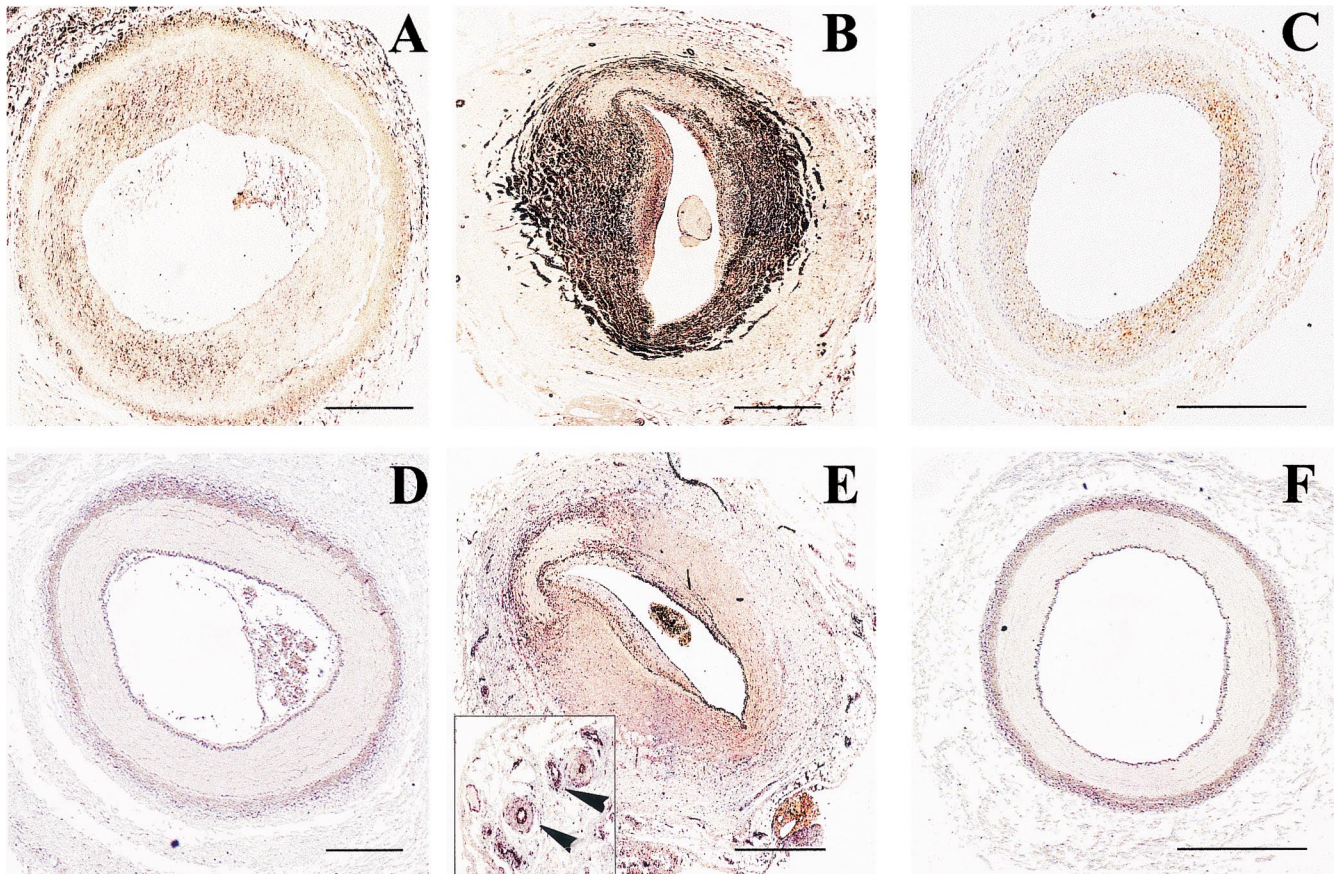


Fig. 1. Immunohistochemical and ISH patterns in vascular sections from hypercholesterolemic pigs. Panels A (NC), B (HC) and C (HCV) show the immunohistochemical distribution of PAI-1 in uninjured iliac arteries after 8 weeks of diet. Panels D (NC), E (HC) and F (HCV) show the ISH pattern corresponding to the same period. Panel E (magnification  $\times 20$ ) also shows an adventitia section with scattered positive cells and strong positive signal in vasa-vasorum endothelial cells (arrowhead). Representative score in the media area: F, 0; C and D, 1; A and E, 2; B, 3 (see text for explanations). Scale bar = 1 mm.

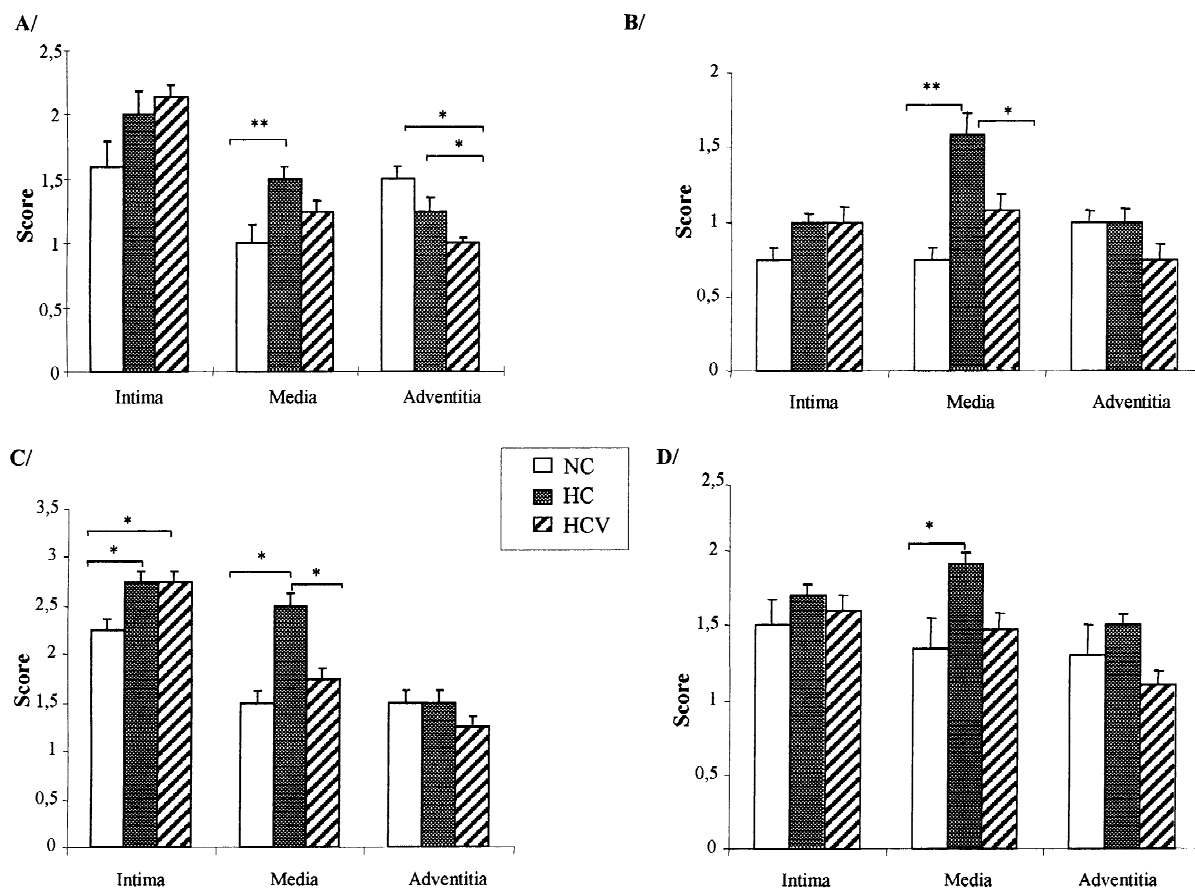


Fig. 2. Semiquantitative assessment of vascular PAI-1 by ISH (panels A and C) and immunohistochemistry (panels B and D) performed after 8 weeks of diet in uninjured iliac arteries (A and B) and in sections taken 28 days after angioplasty (C and D). \* $P<0.05$  and \*\* $P<0.01$ .

throughout the study (Fig. 3A,B,C). An increase in cell density was also observed 28 days after injury (Fig. 3C). The number of vessels with intimal thickening was higher in this group (50% in NC vs. 87.5% in HC,  $P<0.01$ ).

### 3.2.2. PAI-1 expression

Immunoreactive PAI-1 protein was abundantly present in the thickened intima and in the media (Fig. 4A,B) in both NC and HC groups 14 days after vascular injury, persisting after 4 weeks, although higher immunoreactivity was observed at earlier times and more arteries were involved in the HC group (62 vs. 31%).

A strong ISH signal for PAI-1 mRNA was found in the deeper layers of the thickened intima in both NC and HC groups, spreading out through the media up to the adventitia layer (Fig. 4D,E). Changes started 7 days after injury and were sustained during the 4-week period after angioplasty. As shown in Fig. 2C,D, stronger ISH and immunohistochemistry signal for PAI-1 was observed in the intima ( $P<0.05$  for the ISH score) and media ( $P<0.05$ ) in the HC group 4 weeks after mechanical injury as compared with NC.

To determine whether the above-mentioned changes could influence circulating levels of PAI-1, the plasma

inhibitor activity was assayed at different time periods in both NC and HC groups. As shown in Fig. 5, a significant increase of PAI-1 levels was observed 7 days after balloon injury in both NC ( $P<0.001$ ) and HC ( $P<0.05$ ) groups with respect to baseline, still remaining elevated at 14 days in the HC group ( $P<0.05$ ).

### 3.3. Effect of vitamins C and E on PAI-1 expression

Plasma vitamin E levels in the HCV group showed a seven-fold increase with respect to HC group ( $14.1\pm 3.6$  vs.  $1.7\pm 0.2$   $\mu\text{M}$ ,  $P<0.001$ ). The vitamin treatment did not influence significantly the cholesterol levels over the whole studied period, but it did have an effect on PAI-1 both in uninjured and mechanically injured arteries.

#### 3.3.1. Uninjured arteries

Vitamins C and E slightly reduced the intima/media ratio ( $0.2\pm 0.1$  vs.  $0.07\pm 0.01$ ) and percentage stenosis ( $23.5\pm 3.9$  vs.  $14.7\pm 3.3\%$ ) after 8 weeks of diet in HCV as compared to HC groups ( $P<0.05$ ). However, as shown in Fig. 1E,F, a weaker signal for vascular PAI in the media and adventitia was observed in the HCV group and a significant reduction of protein in the media ( $P<0.05$ ) and

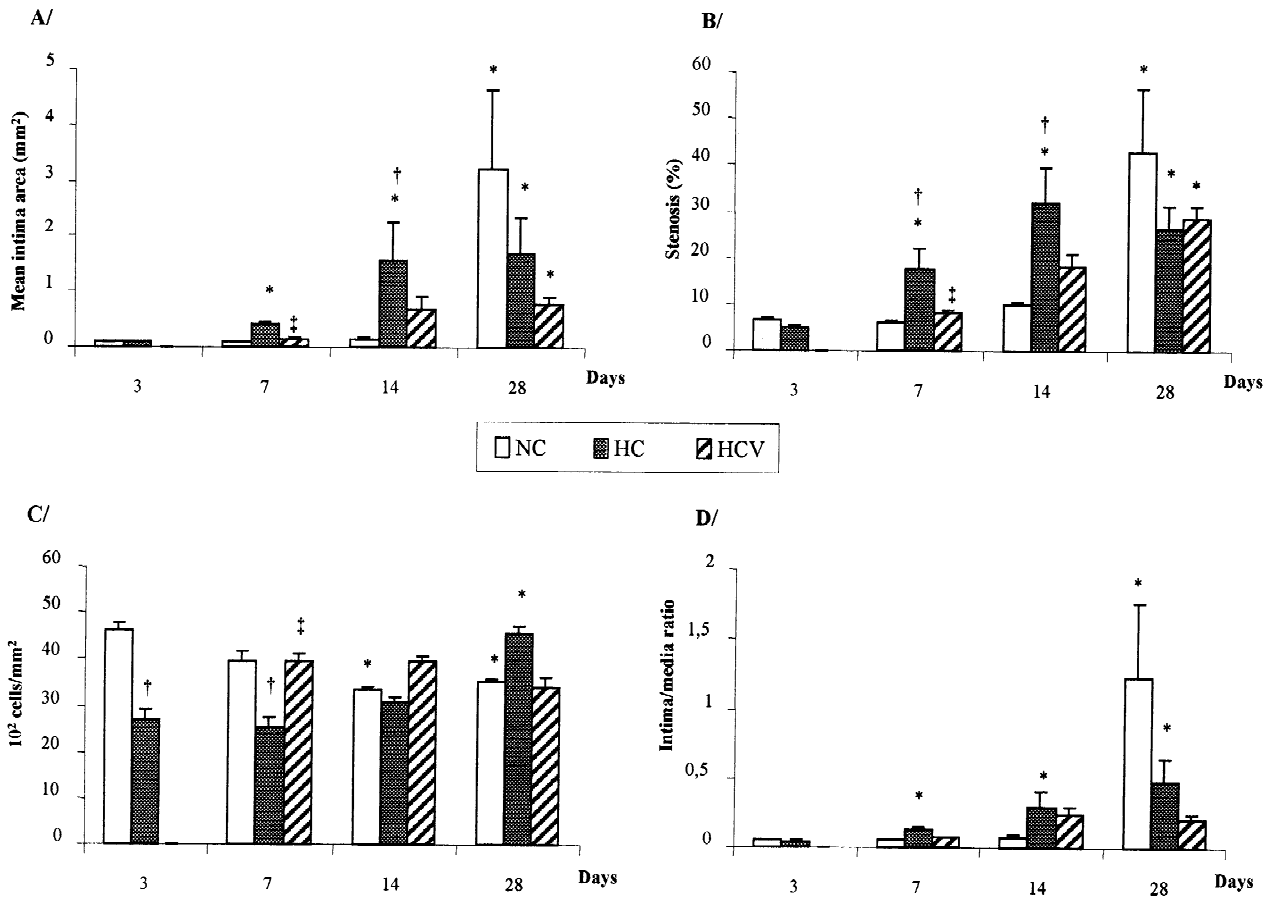


Fig. 3. Morphometric analysis of vascular sections after balloon injury in the different groups of animals. The intima area (A), percentage stenosis (B), number of media cells (C) and intima/media ratio (D) in the balloon injured iliac arteries of NC, HC and HCV minipigs ( $n=4$ ) at the time animals were sacrificed are shown. \* $P<0.05$  as compared to baseline samples, † $P<0.05$  as compared to NC group and ‡ $P<0.05$  as compared to HC group.

mRNA expression in the adventitia ( $P<0.05$ ) was demonstrated in the HCV as compared with HC group at 8 weeks (Fig. 2A,B). Moreover, the number of arteries in HCV group with high PAI-1 expression was lower than in HC animals (25 vs. 16%). In contrast, vitamins did not have any effect on plasma PAI-1 activity in the treated group ( $28.6\pm 0.2$  vs.  $27.5\pm 0.2$  AU/ml).

### 3.3.2. Injured arteries

The vitamin treatment did not improve significantly the mechanical-induced structural changes in the vascular wall (Fig. 3B,C,D), but diminished (30% reduction,  $P<0.05$ ) the mean intima area in relation to HC group (Fig. 3A). Immunohistochemical and ISH patterns for vascular PAI-1 expression (Fig. 4C,F) as well as semiquantitative assessment (Fig. 2C, D) showed weaker PAI-1 expression in the media layer in the HCV group as compared to HC ( $P<0.05$  for the ISH analysis), the score being similar to that observed in control animals. The number of arteries with high PAI-1 expression was also lower in HVC than in HC group (41 vs. 62%). However, vitamins C and E were not able to modify the intima PAI-1 vascular expression induced by hypercholesterolemic diet.

An interesting finding was the highly significant reduction ( $P=0.018$ ) in the systemic PAI-1 activity in the HCV group 28 days after balloon injury with respect to levels at 14 days, reaching the values observed before vascular lesion (Fig. 5).

## 4. Discussion

The main finding of the present study is that a combined treatment of vitamins C and E attenuated the vascular PAI-1 expression and significantly reduced the circulating inhibitor levels induced by angioplasty in hypercholesterolemic pigs, despite only trivial morphologic improvement in this experimental model of mild atherosclerosis.

Evidence is accumulating which indicates that abnormal expression of the fibrinolytic system may promote the development of atherosclerosis and play a role in the remodeling process after vascular injury by influencing vascular SMC migration/proliferation and extracellular matrix accumulation [8,14,18].

The pig model was chosen because of the similarities to the human coronary circulation, spontaneous development



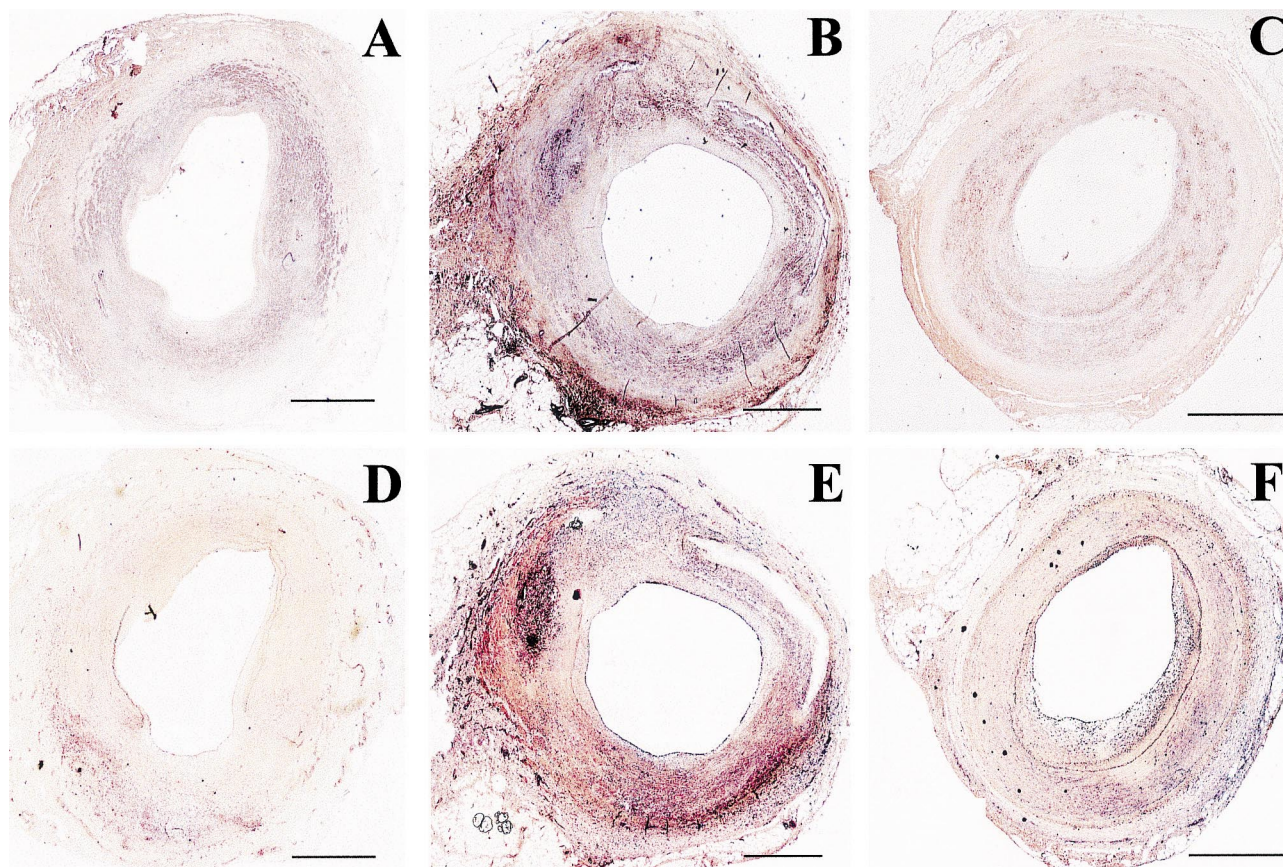


Fig. 4. Vascular PAI-1 expression after angioplasty in normal, hypercholesterolemic and vitamin-treated pigs. Immunohistochemistry (A,B,C) and ISH (D,E,F) patterns in iliac arteries 28 days after angioplasty in NC (A,D), HC (B,E) and HCV (C,F) groups. A strong signal was observed after vascular injury throughout the vessel wall with a marked attenuation in the HCV group. Semiquantitative score in the media area: D, 1; A,C,F, 2; B,E, 3 (see text for explanations). Scale bar=1 mm.

of atherosclerosis and histological response to vascular injury [33].

The present study shows that in the absence of mechanical stress, hypercholesterolemic diet was associated with some morphological changes in the vascular arteries of miniature pigs, indicating a mild degree of atherosclerosis after 4 weeks of diet. These changes were associated with a homogeneous increase in vascular PAI-1 expression in the intima and media areas, as previously reported in different experimental models of hypercholesterolemia [28,34,35] and in human atherosclerotic plaques, suggesting a local involvement of this fibrinolysis inhibitor [18,19,36,37]. Clinical and epidemiological studies have also demonstrated that the elevation of PAI-1 levels is common in several types of atherothrombosis [17,38]. Although the relationship between hypercholesterolemia and thrombosis related to PAI-1 is presently unknown it is worthwhile pointing out that some cholesterol-lowering drugs inhibited PAI-1, resulting in reduced thrombosis formation [39]. The combination of mechanical injury plus hypercholesterolemia was associated with a significant increase in the neointima formation and percentage stenosis, leading to intimal thickening. PAI-1 mRNA and

protein were significantly induced after injury as compared with uninjured arteries, the expression being more intense in the area around the intimal thickening. Thus, vascular injury elicits increased genetic expression of PAI-1 in several components in the induced lesions, which is potentiated by hypercholesterolemia. The intramural PAI-1 related inhibition of proteolysis could play a role in the vascular remodeling process after vessel wall injury [11]. Moreover, circulating levels of PAI-1 were also enhanced after balloon injury, suggesting systemic inhibition of the fibrinolytic activity in response to injury [17,28,38].

We, therefore, tried to assess whether vitamins C and E would be able to modify the PAI-1 expression induced by either hypercholesterolemic diet or after vascular injury. Our results show that vitamin treatment, despite a minimum effect on the morphologic parameters analyzed, reduced the vascular PAI-1 expression induced by hypercholesterolemia and, to a lesser extent, the mechanically induced inhibitor expression. Oxidative stress is increasingly being recognized as a potentially important contributor to atherogenesis and restenosis after vascular intervention and injury [40,41]. The generation of reactive oxygen species and oxidation of lipids have profound and

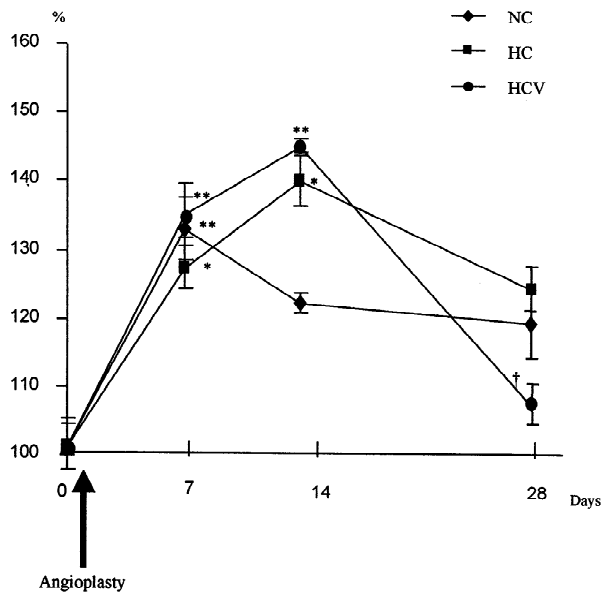


Fig. 5. Plasma PAI-1 activity in pigs after vascular injury. A significant increase was observed in all groups 7 and 14 days after lesion in relation to baseline, with recovery at 28 days especially in the vitamin-treated group. Values are expressed as % of baseline.  $**P < 0.001$ ,  $*P < 0.05$  as compared to values before injury.  $\dagger P = 0.018$  as compared to values obtained 14 days after lesion.

wide-ranging effects that can dramatically increase vascular toxicity and initiate a cascade of molecular and cellular responses [42,43]. Several experimental models using different antioxidants have reported that these agents can slow the progression of atherosclerosis [22–24]. It has been postulated that vitamins C and E exert their inhibitory effect on atherosclerotic lesion development mainly through their antioxidant properties, although they can also decrease protein kinase C, platelet activation and inhibit SMC proliferation [44,45]. Our results suggest that an increase in plasma vitamin levels also influences the endothelial control of fibrinolysis via reduction of vascular PAI-1 in this animal model of angioplasty, although the exact mechanism of action is presently unknown. It has been demonstrated in endothelial cell cultures that PAI-1 secretion shows a dependency on the degree of oxidation that could be completely blocked with the antioxidant probucol [46]. In the clinical setting, the use of antioxidants has proved to ameliorate the metabolic abnormalities and decrease PAI-1 synthesis in renal transplant recipients [47]. Interestingly, vitamin treatment also reduced significantly the angioplasty-induced systemic increase of inhibitor activity suggesting that antioxidants can also regulate fibrinolysis within the circulation. This previously unreported effect seems to be independent of lipid modifications since antioxidants did not alter the plasma cholesterol levels. It can, therefore, be speculated that the reduction of PAI-1 induced by vitamins C and E could be related to their effects improving endothelial function. Interventions aimed at restoration of PAI-1 have

proved to be beneficial to patients with atherosclerotic disease [24].

Because the fibrinolytic system appears to play a significant role in thrombosis and atherosclerosis, specific modulators of the components of the fibrinolytic system are promising targets for therapies of vascular disease. The reported reduction of vessel-wall PAI-1 expression both in injured and uninjured arteries of hypercholesterolemic animal in response to vitamins C and E might represent another step in the beneficial effect of antioxidants in atherosclerotic disease [48].

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