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Antithrombotic effects of saratin on human atherosclerotic plaques

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New Technologies and Diagnostic Tools

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Summary

Platelets play a primary role in thrombus formation after plaque rupture. Platelets recognize the exposed collagen via Von Willebrand factor (VWF) and become activated. Saratin, an inhibitor of the VWF-dependent binding of platelets to collagen, may reduce the thrombotic risk associated to atherosclerosis. Our objective was to evaluate the antithrombotic effects of local treatment with saratin on human atherosclerotic lesions. Thrombus formation was assessed by the deposition of ^{111}In-platelets on different human atherosclerotic lesions under three local shear conditions (800,1700 and 3400/s) with blood derived from catheterized pigs. Human atherosclerotic lesions were locally treated with saratin (30 µg/ml) at 37°C for 5 min and placed in the chamber. Under stenotic shear conditions of 800/s, saratin significantly ($p<0.05$) reduced platelet deposition triggered by human denuded vessel wall (44%), fatty streaks (47%), severely damaged vessel (50%) and atherosclerotic plaque (57%). Thrombus characterization by immunohistochemistry showed also a reduction in fibrin deposition in treated vessels. These results suggest that the local site-specific treatment with saratin inhibits atherosclerotic plaque thrombogenicity at haemodynamic conditions typical of moderately stenotic coronary arteries.

Keywords
Von Willebrand Factor, collagen, saratin, platelets, thrombosis

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Introduction

Platelets play a primary role on thrombus formation after plaque rupture and in the onset of the acute coronary syndromes (1-3). Although the characterization of the initial triggers of thrombosis would significantly improve the efficacy of platelet-inhibitory drugs, the contribution of the different plaque components to thrombosis is not completely understood. Aspirin is the gold standard in clinical antithrombotic therapy but recently new specific antiplatelet agents, such as GPIIb/IIIa antagonists, ADP receptor blockers and specific thrombin inhibitors have been introduced (4, 5). On the other hand, inhibitors of platelet adhesion are not yet in clinical trials, although some have been characterized and tested in purified systems. Platelet adhesion is mediated by Von Willebrand factor (VWF) which acts as a bridge between platelet receptors and collagen (6, 7). Under high shear rate conditions VWF (domain A3) (8) binds to collagen type I and/or III undergoing a conformational change which will allow its binding to the platelet receptor GPIb/IX/V (domain A1) (9). After the initial tethering, platelets will become irreversible adhered through the collagen receptors (GPIa/IIa, GPVI, GPIV, p65, TIIICBP) (10) resulting in platelet activation, degranulation and aggregation (11). Activated platelets will release tromboxane A2, cytokines, mitogenic mediators and vasoconstrictor substances (3) that will lead to a reduction of vascular lumen, thrombus formation and contribute to restenotic complications (12). Therefore, prevention of the initial platelet adhesion step might contribute to a more efficient inhibition of the platelet response to injury in the vessel wall. However, there is not much information on the contribution of the VWF-collagen pathway to thrombosis on human atherosclerotic lesions.

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Saratin (Merck, KGaA) is a 12000 Da recombinant protein (103 aminoacids with three disulfide bridges) isolated from the saliva of the medicinal leech *Hirudo medicinales*, that site-specifically inhibits collagen (type I and III) interaction with VWF-A3 domain (13). Sequential assignment and secondary structure of saratin has been described (14). Saratin has shown to reduce platelet adhesion, hyperplasia (15), and lumen stenosis in carotid endarterectomized rats (16). Up to now saratin has shown platelet inhibitory effects on thrombosis triggered by purified proteins and damaged healthy vessel wall. Therefore, we hypothesized that saratin could also reduce platelet deposition triggered by a highly thrombogenic surface as it is the atherosclerotic plaque. Our results indicate that the local treatment with saratin (i.e. in drug-eluting stents) may reduce thrombosis associated to exposure of different human atherosclerotic lesion components, at shear rates compatible with dilated/stented stenosis in coronary arteries.

Materials and methods

Experimental model

Normal pigs (Large White x Landrace) obtained from a local single farm (n=20; body weight = 38 kg) were individually caged in a light, temperature (23 ± 2°C), and humidity controlled environment with free feeding (normal pig chow) and access to water. Animals were housed for one week before any experimental procedure, to eliminate the stress effects of transportation. All procedures in this study were performed in accordance with institutional guidelines and adhered to the American Physiological Society guidelines for animal research.

Experimental procedure

The pigs were sedated with an intramuscular injection of 8 mg/kg of Azoperona (Stressnil®, Esteve, Spain), deeply anesthetized by intravascular infusion of pentobarbital sodium solution (10 mg/kg, B. Braum, Germany), and then intubated and ventilated (Dog ventilator, Ugo basile, Italy). Through a neck incision, the carotid artery and contralateral jugular vein were cannulated. Blood samples were collected for baseline determination of hematocrit (30.7 ± 0.62%), platelet number (459 ± 13 $\times 10^6$plt/µl), mean platelet volume (56 ± 0.4 μ^3), prothombin time (PT; 12.4 ± 0.1 s), activated partial thromboplastin time (aPTT; 61 ± 8.1 s), and fibrinogen (198 ± 3 mg/dl). Pigs were intravenously heparinized with a bolus (50 U/kg) followed by an infusion (50 U/kg/h) (Liquemine®, Roche, Switzerland). The catheterized carotid artery was connected by polyethylene tubing to the input of the Badimon perfusion chamber (17) and the output of the chamber was connected to a peristaltic pump (Masterflex, Model 7518-10, USA). Blood that passed through the chamber was recirculated back into the animal by the contralateral jugular vein. Each pig was used for about 10 perfusions. The aorta specimens (30 mm in length and 10 mm in width) were placed in the perfusion chamber in a lateral position, forming part of the blood channel by which the test surface was directly exposed to the blood. The substrates were perfused with PBS solution at 37°C for 60 s. After the preperfusion period blood entered the chamber (internal diameter 1.0 mm) at a preselected flow rate of 5, 10 or 20 ml/min for 5 min to obtain a broad range of wall shear rates to encompass conditions in mild stenotic coronary vessels (800/s) and at higher shear rates (1700 and 3400/s) as those described for VWF-dependent-thrombosis in purified substrates or in the microcirculation. These latest high shear rates are not common in the coronaries because of the compensatory distal vasodilation when vessel diameter becomes reduced by high grade stenosis (18). At the end of blood flow, buffer was again passed for 30 s through the chamber under identical flow conditions. The number of deposited platelets on each specimen was normalized from the platelet count, the ^{111}In-activity on the perfused area and in blood, and the area of exposed surface (17).

Preparation of vessel segments and local drug treatment

Pig aorta substrates

A series of experiments were designed to gain information about the dose of saratin required to locally inhibit mural platelet deposition triggered by porcine damaged vessel wall. Pig aortas were obtained fresh, immediately cleaned from adventitia, cut in long pieces, and frozen at −80°C until needed. Before starting the experiments, the aortas were thawed in PBS at 4°C, opened longitudinally, and cut into 30×10 mm segments. Segments of pig aorta were denuded (model of erosion) or severely damaged (model of disruption) by peeling off the intimal layer with a thin portion of subjacent media (19, 20).

Substrates (N ≥10 for each condition) were incubated at 37°C with saratin at different concentrations (0, 3, 30 and 300 µg/ml saratin) for 5 min. The solvent for saratin was PBS which was also used to incubate control tissues. Blood was perfused through the chamber at high shear rate conditions (800 and 1700/s).

Human atherosclerotic substrates

Human aorta specimens were obtained from autopsy cases within 13 to 15 h of death (un-used tissues from an on-going study on sudden death), transported in PBS and immediately cleaned from adventitia, cut in long pieces and frozen at −80°C until needed. Before the experiments, the aortas were thawed in PBS at 4°C, opened longitudinally, and cut into 30×10 mm segments. The specimens were composed of denuded vessels (eroded, N=46), fatty streaks (N=60), severely damaged substrates (disrupted vessel; N=93), and atherosclerotic plaques (N=28). Human specimens devoid of endothelium were classified as denuded/eroded vessels and severely damaged lesions

were obtained by stripping of the intima (model of disruption) as described for the porcine aorta (18, 19). Special care was taken to avoid gross irregularities on the surface. Lesions macroscopically characterized by raised yellow streaks were classified as fatty streaks, while lesions macroscopically characterized by raised white or yellow-white plaques were classified as atherosclerotic plaques. Substrates for each experiment were used in a randomized fashion. Human specimens were incubated in PBS solution with or without saratin (30 µg/ml) at 37°C for 5 min. Blood was perfused at different shear rate conditions (800, 1700 and 3400/s).

Platelet interaction with collagen under elevated shear

We performed additional flow chamber perfusion studies using collagen-coated plastic slides, following previously published methods (21), to assess the inhibitory effect of saratin on platelet-collagen interaction under high shear rate conditions. For this purpose, Permanox® plastic slides (30×10 mm) were coated with fibrillar collagen from bovine Achilles tendon (at a concentration of 16 µg/ml). The plastic slides were kept in a humid atmosphere overnight, then washed, and blocked with 3% BSA for 2h. PBS solution (with or without saratin 30 µg/ml) was added for 5 min at 37°C. Collagen-coated surfaces were placed in the perfusion chamber and perfused as described above at shear rates of 800 and 3400/s. Commassie blue stain (protein) showed a uniform coverage of collagen in the plastic slides. Furthermore, staining of the collagen-coated slides after blood perfusion showed that the collagen matrix had not been dislodged or peeled off by the flow.

Radioactive labeling of platelets

Approximately 24 hours before the perfusion experiment, autologous platelets were labelled with ^{111}In-oxine (^{111}In) (Amersham, Germany) as previously described (20). In brief, 43 ml of blood were withdrawn in 7 ml ACD solution (0.8% citric acid, 2.2% trisodium citrate, 2.45% dextrose, pH 5). Platelets were isolated by low speed centrifugation (400*g*, 10 min), resuspended in ACD-saline (14.4% ACD solution in saline, pH 6.50) and ^{111}In-labeled with ^{111}In. An average of $7.3 \times 10^6 \pm 0.12 \times 10^6$ per µl of ^{111}In-labeled platelets was reinjected in a final volume of 4 ml of autologous plasma. Efficiency was 96 ± 1.2% and the injected activity was 249 ± 9 µCi. The labeling procedure was performed approximately within 2 hours.

Biodistribution of indium-111-labeled platelets and biochemical analysis

At the end of the perfusion experiment post-mortem ^{111}In-biodistribution indicated a correct platelet distribution with maximal accumulation in blood (47 ± 4% in blood, 28 ± 3% in liver, 14 ± 2% in spleen, 4.0 ± 0.5% in lungs, 0.20 ± 0.03% in kidneys, and 0.11 ± 0.02% in heart tissue). Serum levels of creatinine (1.00 ± 0.04 mg/dl), cholesterol (73.0 ± 4.0 mg/dl), protein (4.91 ± 0.05 g/dl), glucose (101.0 ± 7.5 mg/dl), AST (22.0 ± 7.0 U/l) and ALT (33.0 ± 2.4 U/l) were measured by routine analytical chemistry assays and all values were within normal range for pig blood. Indium release from platelets was analyzed throughout the experimental perfusion period and it was always <4%.

Conventional histology and immunohistochemistry in human atherosclerotic substrates

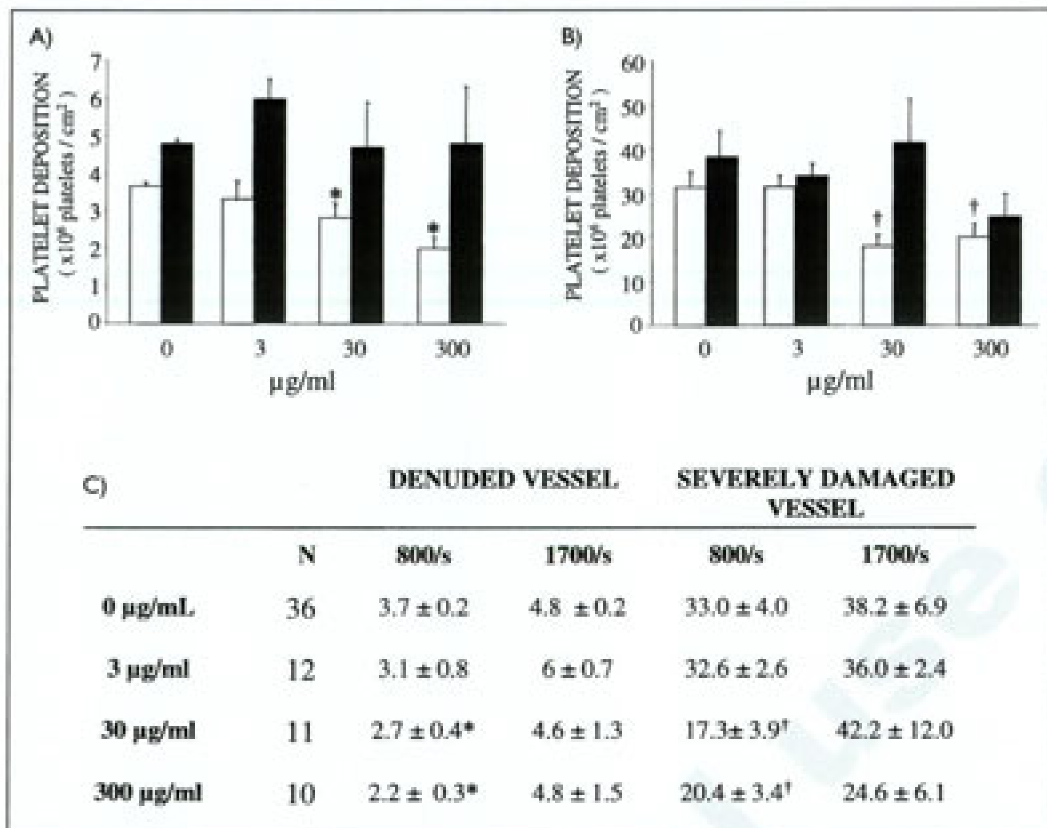
Perfused substrates were fixed in 4% paraformaldehyde, cryoprotected with 2.3 M sucrose and frozen over dry ice in OCT (Tissue-Tek OCT Compound 4583, Leica, Germany). Serially cut 4- to 5 µm sections were obtained from the centerline of the vessel, longitudinal to the blood flow direction (Lung CM 300 Cryostat, Leica, Germany). Sections were mounted on gelatinized slides for immunohistochemistry or conventional staining and stored at –20°C until tested. Masson's Trichromic staining allowed visualization of the different atherosclerotic lesions. For immunohistochemical analysis, antifibrinogen polyclonal antibody (DAKO code No.A=080, Denmark) and an anti-platelet polyclonal antibody (pabBP19) produced in our laboratory (20) were used as primary antibodies. Secondary antibodies were FITC-conjugated F (ab') 2 fragment of anti-rabbit polyclonal (Sigma, code No.F1262, USA) and TRITC-conjugated swine anti-rabbit immunoglobulins (DAKO No.R156, Denmark). Images were captured with an Olympus Vanox AHBT3 microscope and digitalized by a Sony 3CCD camera. Controls of primary and secondary antibody staining were always performed. Control and treated vessels were analyzed from the same axial segment to avoid interference of location in comparative analysis.

Hematological measurements

After each sequence of perfusions, blood samples were collected from each animal and evaluated for platelet count, hematocrit, red blood cells count, fibrinogen levels, prothrombin time, activated partial tromboplastin time, and indium-release from platelets.

Statistical analysis

Results are expressed as mean ± SEM unless otherwise stated. Statistical significance of overall differences between groups was analyzed by analysis of variance (ANOVA) and by Mann-Whitney U test. A Power Macintosh computer equipped with Statview™ software (Abacus, Inc) was used for all analysis. Values of $p<0.05$ were regarded as statistically significant.



C)

	N	DENUDED VESSEL 800/s	DENUDED VESSEL 1700/s	SEVERELY DAMAGED VESSEL 800/s	SEVERELY DAMAGED VESSEL 1700/s
0 µg/mL	36	3.7 ± 0.2	4.8 ± 0.2	33.0 ± 4.0	38.2 ± 6.9
3 µg/ml	12	3.1 ± 0.8	6 ± 0.7	32.6 ± 2.6	36.0 ± 2.4
30 µg/ml	11	2.7 ± 0.4*	4.6 ± 1.3	17.3± 3.9†	42.2 ± 12.0
300 µg/ml	10	2.2 ± 0.3*	4.8 ± 1.5	20.4 ± 3.4†	24.6 ± 6.1

Figure 1: Patelet interaction with damaged pig vessel wall. A) Platelet deposition triggered by denuded pig vessel wall (* $p<0.05$ vs. untreated). B) Platelet deposition triggered by severely damaged pig vessel wall († $p<0.01$ vs. untreated). C) Table of results. Results are expressed as mean values of platelet deposition ($\times10^6$ platelets / cm²) ± SEM. (Open bars, 800/s; hatched bars, 1700/s).

Results

Efficacy of saratin on thrombus formation: dose-finding studies

The antithrombotic effects of saratin were tested under well defined shear conditions and surface thrombogenicity.

Denuded pig vessel wall (model of erosion)

Platelet deposition triggered by denuded vessel wall was significantly inhibited at 800/s with local saratin treatment (30 µg/ml and 300 µg/ml) after 5 min incubation at 37ºC ($p<0.05$) (Fig. 1A). Platelet deposition was reduced from 3.7 ± 0.2 $\times10^6$ platelets/cm² (non-treated) to 2.7 ± 0.4$\times10^6$ platelets/cm² (30 µg/ml saratin; ≈27% reduction vs. non-treated) or to 2.2 ± 0.3$\times10^6$ platelets/cm² (300 µg/ml saratin; ≈40% reduction vs. non-treated). No significant differences were observed at higher shear rate.

Severely damaged pig vessel wall (model of disruption)

Platelet deposition triggered by severely damaged vessel wall was significantly reduced at shear rate of 800/s with saratin at concentrations of 30 and 300 µg/ml ($p<0.01$) (Fig. 1B). At a local saratin treatment of 30 µg/ml the maximal inhibitory effect was reached, with a reduction of ≈52% (33.0 ± 4.0 vs. 17.3 ± 3.9 $\times10^6$platelets/cm²). There were no significant effects at 1700/s.

Thrombus formation on human atherosclerotic substrates

The pattern of thrombus formation on human atherosclerotic lesions was directly regulated by local rheological conditions and tissue characteristics (Fig. 2). Human denuded vessel or fatty streaks showed less platelet deposition than severely damaged or atherosclerotic plaque substrates. Platelet deposition on human denuded vessel, fatty streaks, severely damaged or atherosclerotic plaque increased with increasing shear rates. Two factor ANOVA (A, shear rate; B, substrate) (Table 1) showed significant effects of substrate on platelet deposition, except for similar platelet deposition in denuded vessel and fatty streak and in severely damaged vessel and atherosclerotic plaque. Effects of shear rate range (800 to 3400/s) were significant in each case. Figure 3 shows the Masson's Trichrome stain of human denuded/eroded vessel (Fig. 3A), fatty streak (Fig 3B and 3E), severely damaged disrupted vessel (Fig. 3C), and atherosclerotic plaque (Fig. 3D).

Effects of saratin on human atherosclerotic plaque

Human atherosclerotic tissues were locally treated with saratin (30 µg/ml) for 5 min at 37° C, and the vessels were mounted in the chamber. Under stenotic shear conditions of 800/s, saratin significantly reduced platelet deposition in all conditions. Reductions with respect to control vessels were 44% on denuded vessel wall, 47% on fatty streaks, 51% on severely damaged vessels, and 57% on atherosclerotic plaque (Table 2) ($p<0.05$). At shear rate conditions of 1700/s saratin treated human substrates only showed a significant platelet reduction in fatty streaks ($p<0.05$). No change in platelet deposition was observed at the higher shear rate (3400/s) at any degree of vascular damage.

Immunohistochemical staining (fibrin: Fig. 4; platelets: Fig. 5) was performed on denuded vessel (thrombotic response similar to that on fatty streaks) and severely damaged vessel wall (thrombotic response similar to that on atherosclerotic plaques). The selected human perfused substrates (A, B: denuded vessel; C, D: severely damaged vessel) showed a mild reduction of fibrin deposition after local treatment with saratin (30 µg/ml) for 5 min in both denuded (Fig. 4B) and severely damaged vessels (Fig. 4D) with respect to control substrates (Fig. 4A and 4C). Denuded human vessels demonstrated minimal platelet deposition (Fig. 5A) while severely damaged vessels induced thrombus formation (Fig. 5C). Platelet deposition after saratin treatment followed the pattern of inhibition already seen in the radioisotopic quantitative analysis (denuded vessel Fig. 5B; severely damaged vessel Fig. 5D).

Effects of saratin on collagen-induced thrombus formation under high shear

Saratin significantly reduced platelet adhesion to collagen-coated slides at high shear rates (800/s, $p=0.048$; 3400/s, $p=0.02$) (Fig. 6). Interestingly, saratin induced a 4-fold reduction on thrombus formation at shear rate of 800/s (16.3 ± 9 vs. 4 ± 2 $\times 10^6$ platelets/cm^2; Fig. 6A) while a 16-fold reduction was achieved at a higher shear rate (3400/s: 83 ± 30 vs. $5 \pm 1.5 \times 10^6$ platelets/cm^2; Fig. 6B). According to these results, saratin-induced inhibition of platelet deposition (% inhibition vs. non-treated) was in agreement with a VWF-dependent effect, increasing with increasing shear rate. It was approximately 75% at 800/s and 94% at 3400/s (Fig. 6C).

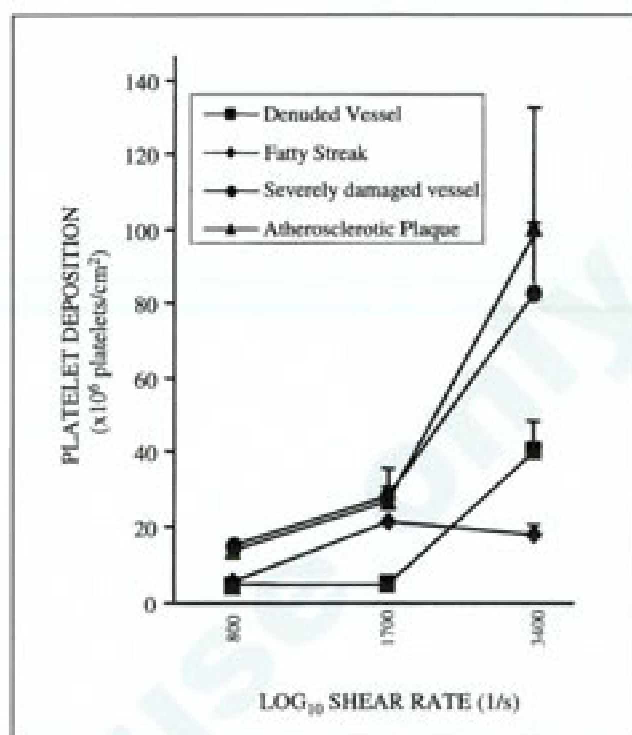


Figure 2: Shear and lesion dependence of platelet deposition on human atherosclerotic lesions. Human denuded/eroded vessel (N= 23) or fatty streaks (N=31) showed less platelet deposition than severely damaged disrupted arteries (N=46) or atherosclerotic plaque (N=14) substrates, and in general platelet deposition increased with increasing shear rates.

Laboratory data

No significant differences were found in hematological parameters before and after starting perfusions of saratin-treated ves-

Table 1: ((■ author: please provide a legend))

COMPARISONS	SHEAR RATE	SUBSTRATE
Denuded vessel vs fatty streak	0.03	N.S
Denuded vessel vs severely damaged vessel	0.005	0.02
Denuded vessel vs atherosclerotic plaque	0.0002	0.004
Fatty streak vs severely damaged vessel	0.01	0.01
Fatty streak vs atherosclerotic plaque	0.0002	0.0006
Severely damaged vessel vs atherosclerotic plaque	0.001	N.S

sels (platelet count: 420.0 ± 10.5 vs. 437.0 ± 12.9 $\times 10^6/mm^3$; red blood cells: 5.40 ± 0.08 vs. 5.60 ± 0.11 $\times 10^6/mm^3$; hematocrit: 30.70 ± 0.30 vs. 30.80 ± 1.00%). Basal coagulation parameters were neither changed after saratin (fibrinogen levels: 201.0 ± 4.1 vs. 205.0 ± 3.4 mg/dl; prothrombin time: 12.7 ± 0.2 vs. 12.5 ± 0.2 s; activated partial thromboplastin time: 227± 35 vs. 232.0 ± 21 s).

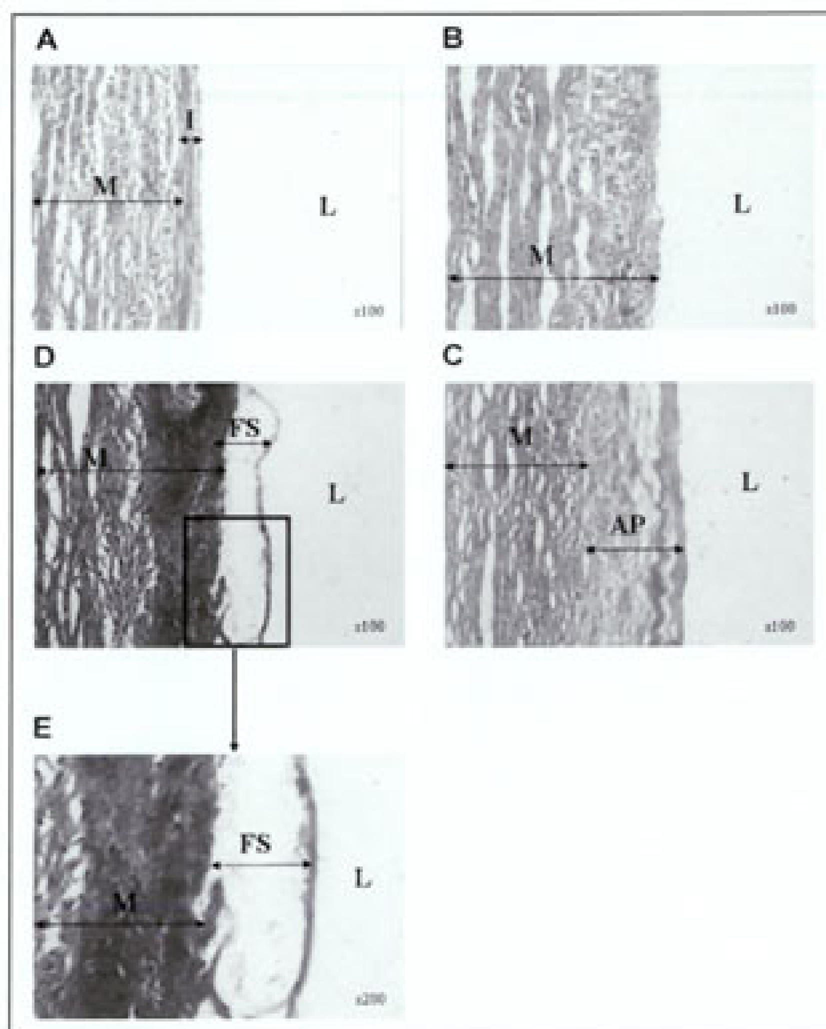


Figure 3: Masson's Trichrome stain of human denuded vessel (without endothelium) (A), fatty streak (B), severely damaged (disrupted exposure of the medial-layer) vessel (C), and atherosclerotic plaque (D) (x100). E, Fatty streak histological photomicrograph at higher resolution (x200). Lipid content (B, D, E) has been lost due to the staining procedures. (L, lumen; I, intima; M, media; FS, fatty streak; AP, atherosclerotic plaque).

Table 2: Platelet interaction with human atherosclerotic components as determined by the radioisotopic (^{111}In-labeled platelets) method under different shear rate conditions. Results are expressed as mean values of platelet deposition ($\times 10^6$ platelets / cm^2) ± SEM. (* $p<0.05$ vs. control).

		DENUDED VESSEL (N=46)		FATTY STREAKS (N=60)		SEVERELY DAMAGED (N=93)		ATHEROSCLEROTIC PLAQUE (N= 28)	
		Control (N=23)	saratina (N=23)	Control (N=31)	saratina (N=29)	Control (N=46)	saratina (N=47)	Control (N=14)	saratina (N=14)
SHEAR RATE (1/s)	800	4.3±0.6	2.5 ±0.5*	5.1±0.7	2.6±0.5*	14.9±2.5	7.6±0.9*	13.8±2.7	6.0±2.2*
	1700	4.8±0.9	4.3 ±0.6	21.1±10	7.0±1.5*	28.4±3.1	23.0±5.0	27.3±8.9	24.3±7.4
	3400	40.2±8.2	38.3±10.1	18.2±4.0	20.1±5.2	82.2±20.4	83.1±23.2	98.9±34.1	51.7±36.6

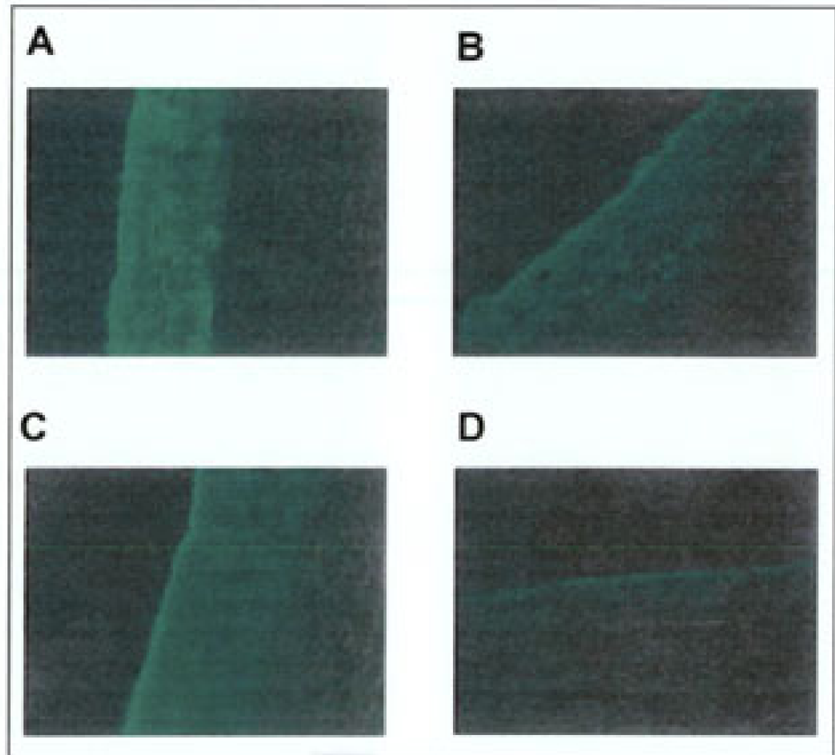


Figure 4: Representative immunophotomicrographs of fibrin deposition under shear rate conditions of 800/s. Non-treated (control) human substrates (A, C) or treated with saratin (B, D). Saratin reduced fibrin deposition. A, B: denuded/eroded vessel; C, D: severely damaged disrupted vessel. (Magnification x200).

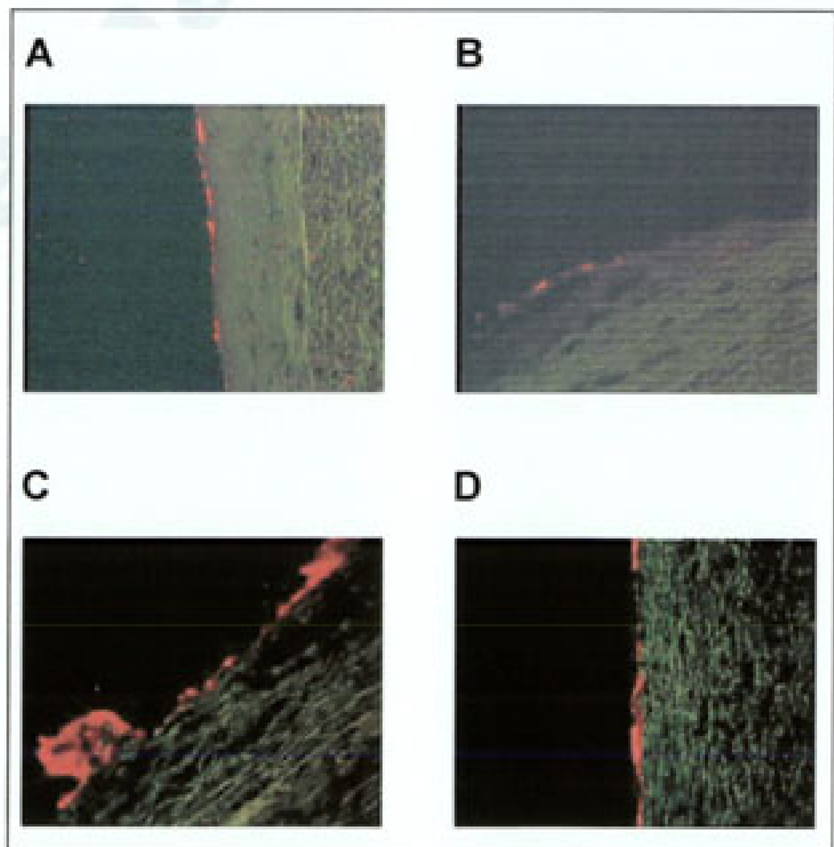


Figure 5: Representative immunophotomicrographs of platelet deposition at shear conditions of 800/s. Non-treated (control) human substrates (A, C) or treated with saratin 30µg/ml (B, D). Saratin reduced thrombus formation. A, B: denuded/eroded vessel; C, D: severely damaged disrupted vessel. (Magnification x200)

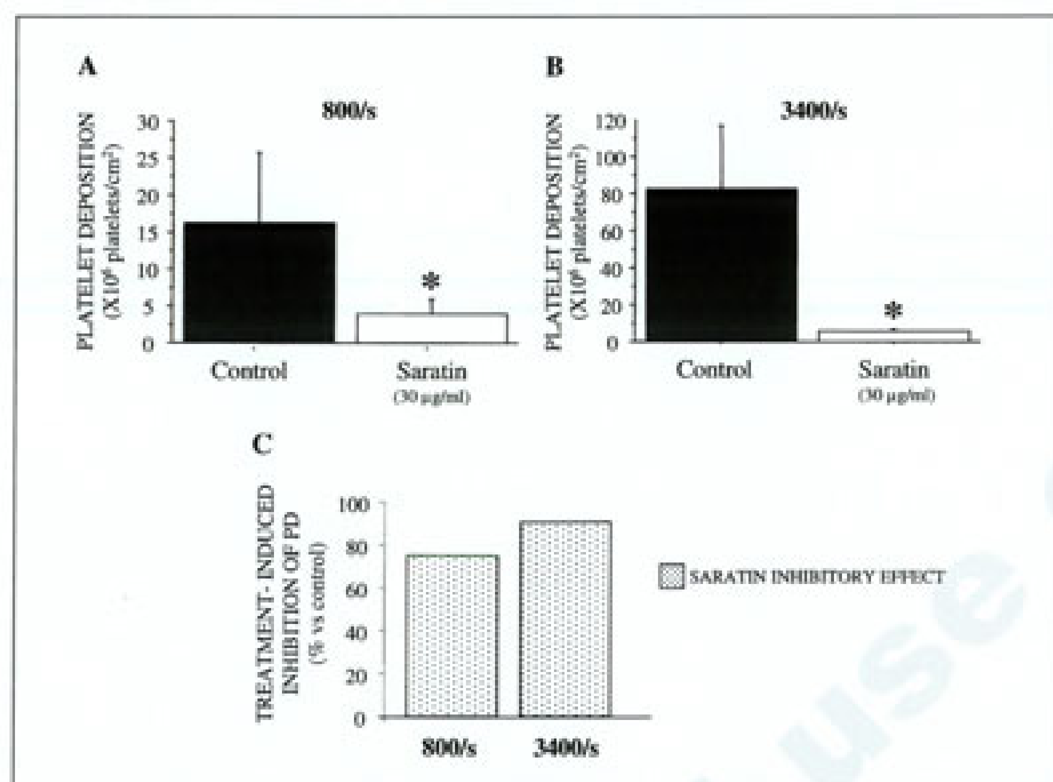


Figure 6: Bar graph of platelet interaction with collagen-coated slides under high shear rate conditions. Results are expressed as mean values of Platelet Deposition (x106 platelets / cm²) ± SEM. Collagen-coated slides were incubated in PBS with (N=16) or withouth (N=16) saratin (30 µg/ml; 5 min; 37°C) at 800 and 3400/s. Saratin-treated collagen slides showed a significant decrease on platelet deposition with respect to controls both at 800/s (A) and 3400/s (B). Treatment-induced inhibition of platelet deposition at 3400/s than 800/s (C).

Discussion

Under high shear conditions (>650/s) (22) Von Willebrand Factor-A3 domain (VWF) plays an important role in mediating platelet adhesion to purified collagen preparations (8). VWF-A3 binding to collagen is followed by a conformational change which will enable VWF-A1 domain/GPIb/IX platelet receptor interaction leading to platelet activation (9). Activated platelets provide a surface for the assembly of the coagulation protein complexes that generate thrombin, and also serve as a nidus for fibrin clots (23). Interestingly, there are no studies on the impact of this hemostatic pathway in arterial thrombosis triggered by atherosclerotic plaques.

Up to now, only a few *in vivo* studies in animal models have reported that inhibition of platelet-VWF interaction is effective in reducing thrombogenesis triggered by injured arteries. These studies include the use of anti-GPIb monoclonal antibodies (MoAb) (24, 25), anti-VWF MoAb (26), a recombinant Von Willebrand factor GPIb binding domain (VCL) (27), and other leech-derived substances as calin (28) or leech antiplatelet protein (LAPP) (29). Although potentially efficacious they have shown some secondary unwanted effects in the animal models and experimental designs studied; as such, anti-GPI MoAbs seem to induce severe thrombocytopenia (25, 30), VCL prolongs bleeding time (31), and local treatment with LAPP has shown no effect on thrombus formation in baboons (29). We show here that local treatment of atherosclerotic human plaques with saratin, seems to reduce their thrombogenicity under certain flow conditions. Saratin, a recombinant protein isolated from the saliva of the leech *Hirudo medicinalis*, had previously shown a potent inhibitory effect on VWF-dependent platelet adhesion to purified collagen at high to very high shear rates (13).

Additionally, Cruz et al (16) demonstrated that topical application of saratina in a normal rat carotid endarterectomy model reduced thrombus formation (low shear rate). In our study, we demonstrate that local treatment of highly thrombogenic atherosclerotic vessels with saratin reduces thrombus formation. Indeed, all damaged human substrates incubated with saratin (30 µg/ml) and perfused at shear rates of 800/s showed a significant reduction on platelet deposition. However, no significant reduction on platelet deposition was observed at higher shear rate conditions (1700 and 3400/s). Interestingly, our data showing saratin inhibitory effects on platelet deposition induced by

collagen-coated surfaces under high shear rates (800 and 3400/s) suggest that saratin remains bound to collagen as already described (13). Therefore, our results with human atherosclerotic and damaged porcine vessels suggest that thrombosis at high shear rates is not as dependent on VWF as it may be the case for purified collagen. Indeed, we have previously shown that on highly thrombogenic surfaces such as atherosclerotic plaque the blockade of the thrombin pathway seems to be highly effective (32).

While on purified collagen monolayers VWF-dependent effects were evident at shear rates typical of the microcirculation (>1000 /s) (29, 33), in pig arterial substrates we have previously shown that VWF effects were also evident at low shear rates typical of larger vessels (200 to 400/s) (34). Therefore, our data perfusing atherosclerotic human vessels support the concept that saratin effects are significant at those shear rates typical of coronary circulation. Indeed, the highest prevalence of thrombus-dependent acute coronary syndromes occur in coronaries that are moderately stenotic (35). Moreover, saratin was more efficacious in diminishing thrombosis induced by highly thrombogenic surfaces (severe plaques and severely damaged disrupted vessels), conditions that are mainly associated to the presentation of the acute coronary syndromes. Immunohistochemical analysis supported the decrease in platelet deposition already seen in the radioisotopic quantitative analysis and additionally showed a decrease in fibrin deposition on denuded and severely damaged vessels.

In conclusion, this study demonstrates that local saratin treatment significantly decreases platelet deposition and mural thrombus formation triggered by human atherosclerotic lesions at hemodynamic conditions typical of moderately stenotic coronary arteries. Saratin treatment may become a successful approach in the local treatment of revascularized vessels with drug-eluting stents because interventional procedures usually expose highly thrombogenic surfaces to circulating blood. Additionally, saratin has previously shown anti-restenotic properties (13) that, in addition to its antithrombotic profile, may be of benefit in revascularization stent-mediated procedures.

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A novel anti-ischemic nitric oxide donor inhibits thrombosis without modifying haemodynamic parameters.

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A novel anti-ischemic nitric oxide donor inhibits thrombosis without modifying haemodynamic parameters

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Summary

Platelets are involved in the clinical presentations of ischemic heart disease. Our objective was to study the antithrombotic effects of a new nitric oxide donor (LA419), a neutral sugar organic nitrate with a protected thiol group in its molecular structure. Animals were randomly distributed in three groups: I) oral administration of LA419 (0.9-1.8-3.6-5 mg/kg/d, 10 days); II) oral administration of standard IS-5-MN (0.9-1.8 mg/kg/d, 10 days); III) non-treated group (control). In catheterized pigs, thrombosis was studied under controlled rheological conditions by radioisotopic evaluation of deposited platelets on damaged vessel wall, placed in an extracorporeal perfusion chamber. Changes in blood pressure, heart rate, and platelet aggregation were evaluated. Results have shown that LA419 significantly decreased thrombus formation according to the degree of vascular damage, and shear rate conditions in a dose-dependent manner ($p<0.005$), without significant modifications on blood pressure and/or elevation of liver enzymes. In contrast, IS-5-MN only showed a significant reduction on platelet deposition at the high dose, that was associated to hypotension and elevation of liver enzymes. Therefore, we conclude that this new anti-ischemic NO-donor (NOd) LA419 that inhibits platelet function without modifying blood pressure may be a highly efficacious strategy to passivate platelet activation induced by a damaged vessel wall.

Keywords

Platelets, thrombosis, nitric oxide, blood pressure, pigs

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Introduction

Atherosclerotic narrowing of one or more coronary artery is responsible for myocardial ischemia and angina in most patients with stable angina pectoris (SAP). The coronary arteries of patients with SAP also contain many non-obstructive plaques, which are prone to fissures or rupture resulting in presentation of acute coronary syndromes (1). At the site of endothelial injury, there is decreased production of the endogenous inhibitors of platelet aggregation and vasoconstriction, hence creating a local prothrombotic environment. Therefore, in addition to symptomatic relief the emphasis of treatment has been to reduce atherothrombosis. Combination therapy of anti-ischemic agents and aspirin, in addition to a reduction in risk factors, provides optimal management in patients with ischemic heart disease (2).

Under physiological conditions, nitric oxide (NO) released from platelets and endothelium is involved in many vascular processes such as vascular smooth muscle cell (VSMC) relaxation and inhibition of platelet adhesion and aggregation (3). The term "nitrovasodilator" has come to be utilized to designate a chemically heterogeneous group of agents that are linked by the fact that they contain at least one biologically active NO moiety, and that their pharmacological effect include dilatation of VSMC. Nitrovasodilators such as nitroglycerin, isosorbide-5-mononitrate (IS-5-MN) and other nitrates belong to the most widely prescribed drug category in ischemic heart disease (4). The vasodilator and antiplatelet effects of nitrovasodilators are

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mediated mainly by an increase in guanosine 3', 5'-cyclic monophosphate (cGMP) level through activation of guanylate cyclase (GC) (5). In patients with SAP, oxidative stress has been reported to induce clearance of platelet and endothelial NO and inactivation of soluble GC (5, 6). Therefore, in patients with SAP, exogenous donation of NO by nitrovasodilators is the strategy nowadays used to restore NO-functions.

Treatment with conventional nitrate preparations is limited by potential hemodynamic effects (hypotension) (7), poor anti-thrombotic properties (8), and drug tolerance (9). To overcome these limitations, development of novel NO donors with anti-thrombotic/anti-ischemic properties and negligible hemodynamic effects has been a challenge for the correct treatment of patients with ischemic heart disease.

In this study, we investigated whether donation of NO with a neutral sugar organic nitrate with a protected thiol group in its molecular structure (LA419) (10) inhibits thrombosis triggered by eroded and severely damaged vessel wall at shear rates typical of patent stenotic coronary arteries in the experimental porcine model. This study indicates that the new nitric oxide donor LA419 in addition to its previously described anti-ischemic properties) (10), has a high efficacy in reducing the risk of thrombotic complications in damaged vessels without modifying blood pressure.

Methods

Experimental procedure and perfusion system

Studies were performed in normal pigs (Large White × Landrace; ≈ 36 kg) housed for a week before any experimental procedure. Pigs (n=31) were sedated by intramuscular injection of 8 mg/kg of Azoperona (Stressnil®, Esteve, Barcelona, Spain), anesthetized by intravascular infusion of pentobarbital sodium solution (10 mg/kg, B.Braum, Barcelona, Spain), and then intubated and ventilated (Dog ventilator, Ugo basile, Italy). To minimize the circulating plasma levels of pentobarbital and avoid barbiturates platelet inhibitory effect, (11) we administered 10 mg/kg i.v. bolus of pentobarbital sodium (deep anesthesia) followed by a continuous infusion of 10 mg/kg/h until the experiment had been completed. This procedure also produced a consistent anesthetic state with a minimal variation in hemodynamic parameters. Through a neck incision the carotid artery (distal portion) and contralateral jugular vein were cannulated. Then, pigs were intravenously heparinized with a bolus (50 IU/kg) followed by an infusion (50 IU/kg/h) (Liquemine®, Roche, Switzerland). All animals received this low-dose anticoagulation with heparin (aPTT ratio = 1.5-2.5) to prevent occlusion in the extracorporeal system. The catheterized carotid artery was connected by polyethylene tubing to the input of the Badimon's perfusion chamber and the output of the chamber was connected to a peristaltic pump (Masterflex, Model 7518-10, Cole Parmer Instrument Company, USA). Blood that passed through the chamber was recirculated back into the animal by the contralateral jugular vein.

Blood drawn into perfusion flow chambers perfused the porcine vessels that were deendothelialized (model of erosion or mildly damaged) or mechanically disrupted by peeling off the intimal layer with a thin portion of subjacent media (model of disruption or severely damaged) as previously described (12). We have selected a flow rate of 10 ml/min in the small (0.1 cm diameter) and large (0.2 cm diameter) chamber. This flow gives theoretically calculated average blood velocities of 21.2 cm/s and 5.3 cm/s respectively (13). These shear rates correspond to values ranging from those of large, healthy (unobstructed) arteries (212/s) to values typical of areas of the atherosclerotic vessels (1680/s) (14). At these shear rates, blood can be considered a Newtonian fluid with constant viscosity (15). Several 5-minute perfusions (≈ 30 perfusions per pig) with varying hemodynamic conditions and triggering substrate were performed in each animal (12, 13). The perfused segments were fixed in 4% paraformaldehyde in PBS, and labeled platelets were counted in a gamma counter (Wizard, Wallac, USA) for quantization of deposited platelets. Values were normalized by blood ^{111}In activity (counts), platelet counts in blood, and area exposed surface (13). All procedures in this study were performed in accordance with institutional guidelines and followed the American Physiological Society guidelines for animal research.

Radioactive labeling of platelets

After overnight fasting, 43 ml of blood was withdrawn in 7 ml of anticoagulant citrate dextrose (ACD) solution by femoral venipuncture. Platelets were isolated by differential centrifugations and labeled with ^{111}In-oxine (^{111}In) (Amersham Biosciences, London, UK), as previously described (12). An average of $8.4\times10^6 \pm 0.1\times10^6$ per µL of ^{111}In-labeled platelets were suspended in a final volume of 4 ml of autologous plasma, and were reinjected intravenously into the pig within 2 hours of starting the labeling procedure. Efficiency of labeling was 95 ± 2.7% and the injected activity was 250 ± 10 µCi.

Post-mortem ^{111}In-biodistribution indicated a correct platelet distribution with maximal accumulation in blood (blood: 62 ± 4%, liver: 21 ± 3%, spleen: 8.8 ± 1.4%, lungs: 3.2 ± 0.5%, kidneys: 0.27 ± 0.05%, heart tissue: 0.08 ± 0.01%).

Drug administration

Pigs were randomly distributed in three groups: one nitrate-control (standard IS-5-MN), one treated with LA419, and one control (non-treated). Pigs were given a dosage of nitrate twice daily (9 a.m.-18 p.m.) over a period of 10 days. The non-treated group was kept under the same conditions for 10 days. In order to evaluate a possible dose-dependent effect and establish relations between compounds, IS-5-MN and LA419 were given at increasing dosages until unwanted side effects appeared. As

starting dose, we selected the maximal recommended therapeutic dose of IS-5-MN for humans. Therefore, IS-5-MN was given at a therapeutic dose of 0.9 (7, 16) and at 1.8 mg/kg, whereas LA419 was given at doses of 0.9, 1.8, 3.6, and 5 mg/kg.

Platelet aggregation

Whole blood (WB)

WB impedance platelet aggregation triggered by collagen (3, 5, 10 µg/mL) (Chrono-Log model 530; ChronoLog, Yzasa SL, Havertown, USA) was measured as previously reported (17). Platelet aggregation was measured the day before starting nitrate administration and on the experimental day (day 11).

Platelet-Rich Plasma (PRP)

Optical platelet aggregation was measured in PRP as previously described (17) with a platelet aggregometer (Aggrecorder II, model PA3220, Menarini Diagnostic, Firenze, Italia) at the same periods as in WB. ADP (3, 5, 10, 15 µmols/l) and collagen (3, 5, 10, 15 µg/ml) were used as agonists.

Hematological, coagulation, biochemical and physiological parameters

Blood cell counts, hematocrit, platelet number, and size distribution were performed with a System 9000 cell analyzer (Serono-Baker Diagnostic Allentown, USA). Levels of PT (prothrombin time), aPTT (activated partial tromboplastina time) and plasma fibrinogen were monitored with an ST4 automated clotter (Diagnostica Stago, Asnières, France). To assess any adverse effects of continuous oral drug administration, blood samples were taken for the measurement of blood urea nitrogen (BUN), creatinine, aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) by routine analytical chemistry assays before and after nitrate treatment (IS-5-MN and LA 419).

Systemic blood pressure and heart rate were monitored by a pressure transducer (Letica SA, Rochester, USA), attached to the cannulated femoral artery. Measurements were taken throughout the experiments to determine nitrate hemodynamic effects.

Determination of platelet and plasma cGMP levels

To determine cGMP platelet and plasma levels we performed a new series of experiments. Pigs (n=4) were given an intravenous infusion of LA419 at doses that caused thrombosis reduction (2.5 mg/kg) (data not shown). Blood was withdrawn in EDTA at different periods (pre-treatment and 30-120 min during intravenous treatment), centrifuged at 250xg 10 min at room temperature (RT) and IBMX (1 mmol/l) was added to PRP to avoid phosphodiesterases activity. Samples were centrifuged at 1.400xg 15 min RT to obtain platelet pellets which were stored deep-frozen (-80ºC) until measurement. cGMP levels were evaluated by a commercially available cGMP enzyme immunoassay (EIA) kit (Amersham, Life Science, Chicago, USA) with the addition of an acetylating step to increase sensitivity.

Immunohistochemistry

Perfused pig arterial segments were fixed in 4% paraformaldehyde, cryoprotected with 2.3 mol/l sucrose and stored in OCT (Tissue-Tek OCT Compound 4583, Germany). Serial cut sections (4-5 µm) in the blood flow direction were analyzed. A double immunohistochemical analysis was performed with an antifibrinogen rabbit-polyclonal antibody (DAKO code No.A=080, Glosatrup, Denmark) and an anti-platelet polyclonal antibody (pabBP19) produced in our laboratory and previously described (18). Secondary antibodies were FITC-conjugated F(ab')2 fragment of anti-rabbit polyclonal (Sigma, code No.F1262, New York, USA) and TRITC-conjugated swine anti-rabbit immunoglobulins (DAKO No.R156, Glosatrup, Denmark), respectively. Results were evaluated with a fluorescence microscope (Vanox AHBT3, Olympus, Melville, USA). The images were digitalized by a Sony 3CCD camera. Controls

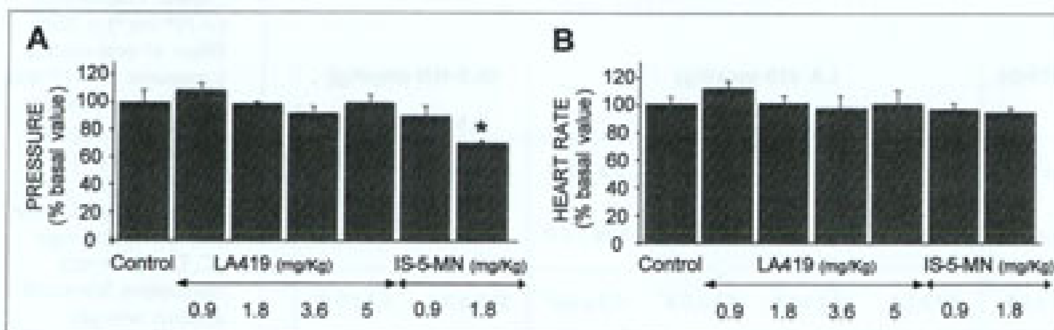


Figure 1: Follow up hemodynamical parameters. Baseline values of mean arterial pressure (A) and heart rate (B) after 10 day oral treatment with IS-5-MN or LA419 vs. control group. (MANOVA + Dunnett's test; *$p<0.05$). (blood pressure: 50.5 ± 4.8 mm Hg; heart rate: 70.0 ± 4.5).

of primary and secondary antibody staining were always performed. Control and treated vessels were analyzed from the same axial segment (centre piece) to avoid interference of location in comparative analysis (19).

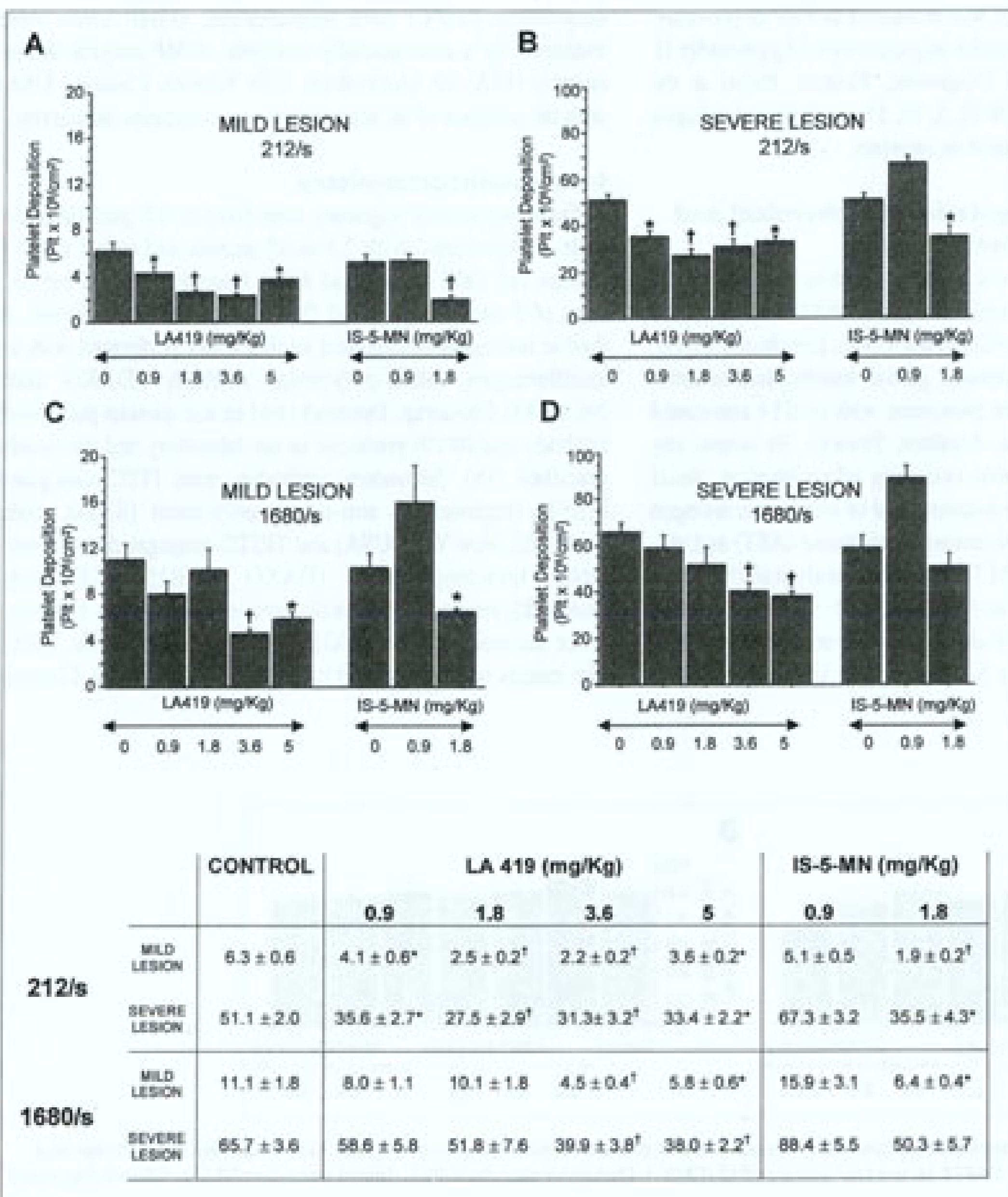
Statistical analysis

All values are expressed as mean ± SEM. Overall differences between groups were analyzed by Analysis of Variance measures (ANOVA). When significant, multiple comparisons were performed by Scheffé's test. Mann-Whitney *U*-test was applied when groups had unequal variances. A p value less than 0.05 was considered significant.

Results

Hemodynamic effects of LA419 and IS-5-MN

No significant differences *vs.* the non-treated group were detected in mean arterial pressure after a 10 day treatment with any of the doses tested for LA419 (Fig. 1A). On the contrary, 1.8 mg/kg of IS-5-MN (standard control) induced a significant drop in mean arterial pressure of 13 mm Hg (≈ 30% reduction *vs.* basal value). No significant changes in heart rate were detected with either LA419, or IS-5-MN within the dose range tested (Fig. 1B).



		CONTROL	LA 419 (mg/Kg)				IS-5-MN (mg/Kg)	
			0.9	1.8	3.6	5	0.9	1.8
212/s	MILD LESION	6.3 ± 0.6	4.1 ± 0.6*	2.5 ± 0.2†	2.2 ± 0.2†	3.6 ± 0.2*	5.1 ± 0.5	1.9 ± 0.2†
	SEVERE LESION	51.1 ± 2.0	35.6 ± 2.7*	27.5 ± 2.9†	31.3± 3.2†	33.4 ± 2.2*	67.3 ± 3.2	35.5 ± 4.3*
1680/s	MILD LESION	11.1 ± 1.8	8.0 ± 1.1	10.1 ± 1.8	4.5 ± 0.4†	5.8 ± 0.6*	15.9 ± 3.1	6.4 ± 0.4*
	SEVERE LESION	65.7 ± 3.6	58.6 ± 5.8	51.8 ± 7.6	39.9 ± 3.8†	38.0 ± 2.2†	88.4 ± 5.5	50.3 ± 5.7

Figure 2: Bar graph and table of platelet-vessel wall interaction as determined by the radioisotopic (^{111}In-labeled platelets) method. Results are expressed as mean values of Platelet Deposition ($\times 10^6$ cm^{-2}) ± SEM. Effect of oral nitrate treatment (LA419 and IS-5-MN) in platelet deposition triggered by mildly (A, C) or severely (B, D) damaged vessel wall under low (A, B) and high (C, D) shear rate conditions. Statistical analysis was performed by analysis of variance (Scheffé's F test; *p<0.05; † p<0.005).

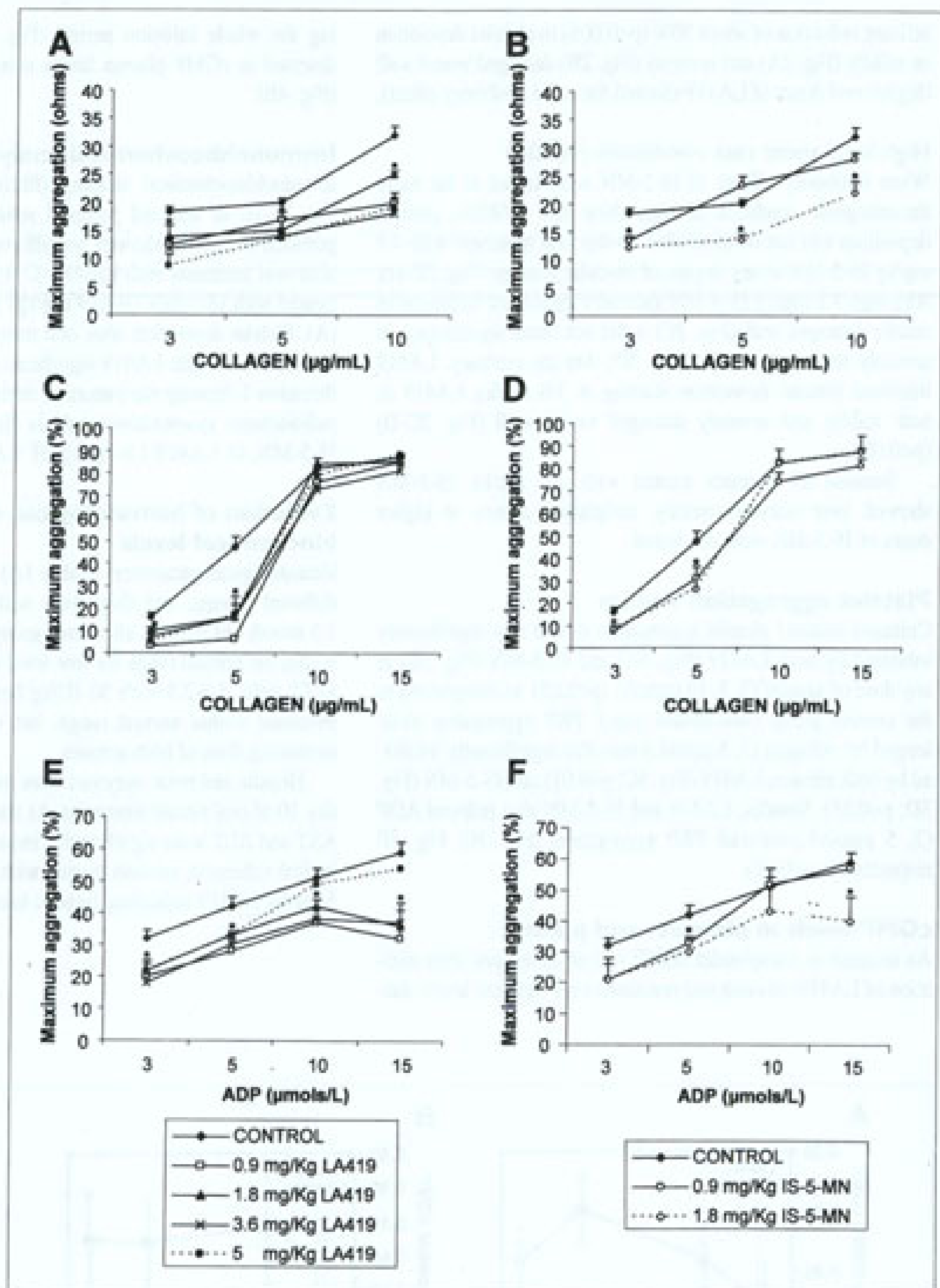


Figure 3: Effect of the different treatments on *in vitro* platelet aggregation. Whole blood (A, B) and PRP (C-F) platelet aggregation induced by collagen (A-D) or ADP (E, F). (Differences were analyzed by Mann-Whitney *U*-test; $^{*}p<0.05$).

Antiplatelet effects of LA419 and IS-5-MN

Low local shear rate conditions (212/s)

Pigs, orally treated with 0.9 mg/kg IS-5-MN, did not inhibit platelet deposition triggered by mildly (deendothelialized) (Fig. 2A) or severely (mechanically disrupted) (Fig. 2B) damaged vessel wall. Pigs treated with an oral dose of 1.8 mg/kg IS-5-MN showed inhibition on mural thrombosis both in mildly (Fig. 2A) ($p<0.005$) and severely (Fig. 2B) ($p<0.05$) injured vessel wall. In contrast, pigs treated with oral doses of 0.9 mg/kg LA419 showed a significant reduction on platelet deposition on both, mildly and severely (Fig. 2A-B) ($p<0.05$) damaged vessel wall. An oral dose of LA419 of 1.8 mg/kg showed a maximal and sig-

nificant reduction of about 50% ($p<0.005$) in platelet deposition on mildly (Fig. 2A) and severely (Fig. 2B) damaged vessel wall (higher oral doses of LA419 showed the same inhibitory effect).

High local shear rate conditions (1680/s)

When inhibitory effects of IS-5-MN were tested at the more thrombogenic condition of high shear rate (1680/s), platelet deposition was not reduced after 10-day oral treatment with 0.9 mg/kg IS-5-MN at any degree of vascular damage (Fig. 2C-D). Although 1.8 mg/kg IS-5-MN showed a significant reduction in mildly damaged wall (Fig. 2C) it did not reach significance in severely damaged vessels (Fig. 2D). On the contrary, LA419 inhibited platelet deposition starting at 3.6 mg/kg LA419 in both mildly and severely damaged vessel wall (Fig. 2C-D) ($p<0.005$).

Because all animals treated with 1.8 mg/kg IS-5-MN showed liver enzyme toxicity, antiplatelet effects at higher doses of IS-5-MN were not tested.

Platelet aggregation studies

Collagen-induced platelet aggregation in WB was significantly inhibited by both LA419 (Fig. 3A) and IS-5-MN (Fig. 3B) at any dose of agonist (3, 5, 10 µg/mL) ($p<0.05$) in comparison to the control group (non-treated pigs). PRP aggregation challenged by collagen (3, 5 µg/mL) was also significantly inhibited by both nitrates, LA419 (Fig. 3C; $p<0.01$) and IS-5-MN (Fig. 3D; $p<0.05$). Besides, LA419 and IS-5-MN also reduced ADP (3, 5 µmols/L)-induced PRP aggregation (Fig. 3E; Fig. 3F respectively; $p<0.01$).

cGMP levels in platelets and plasma

An increase in intraplatelet cGMP was observed just after initiation of LA419 infusion and remained over baseline levels during the whole infusion period (Fig. 4A). No variation was detected in cGMP plasma levels compared to baseline levels (Fig. 4B).

Immunohistochemical analysis

Immunohistochemical staining (fibrin: Fig. 5A-D; platelets: Fig. 5E-H) of selected perfused substrates (disrupted vessels perfused at 1680/s) showed no differences on fibrin deposition after oral treatment with LA 419 (C: 1.8 mg/kg; D: 5 mg/kg) or treated with IS-5-MN (B: 0.9 mg/kg) *vs.* the non-treated group (A). Platelet deposition after oral treatment with IS-5-MN was not inhibited while LA419 significantly reduced platelet mural thrombus following the pattern of inhibition already seen in the radioisotopic quantitative analysis (E: control; F: 0.9 mg/kg IS-5-MN; G: LA419 1.8 mg/kg; H: LA419 5 mg/kg).

Evolution of hematological, coagulation and biochemical levels

Hematological parameters (Table 1A) were similar among the different groups, and they were within normal intervals for 1.5 month old pigs. In all animal groups, aPTT mean ratio was within the normal range for low level of anticoagulation (mean aPTT ratio: 1.5-2.5 with 50 IU/kg heparin). Fibrinogen levels remained within normal range, but showed a decrease with increasing dose of both nitrates.

Hepatic and renal enzymes were measured before and at the day 10 of oral nitrate treatment. As shown in Table 1B, plasma AST and ALT were significantly increased (exceeding physiological values) in animals treated with 1.8 mg/kg IS-5-MN and 5 mg/kg LA419 indicating hepatic toxicity.

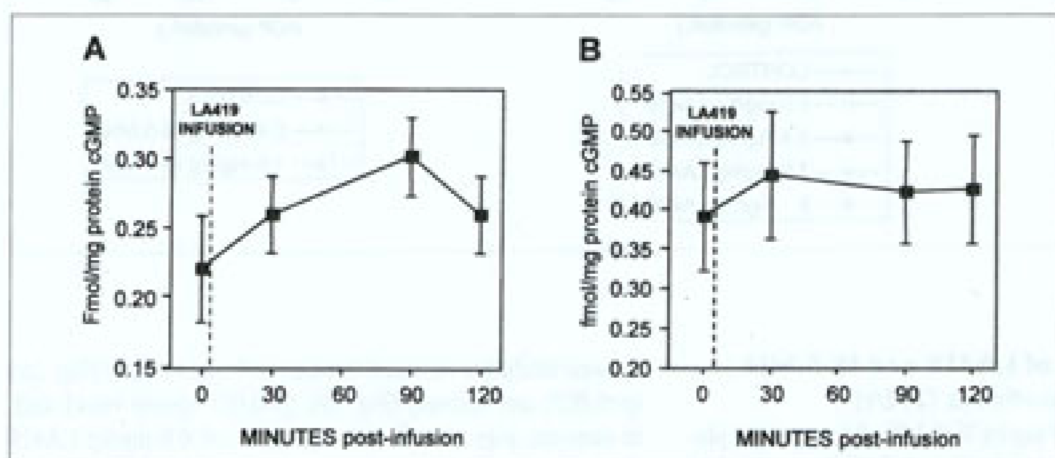


Figure 4: cGMP levels. cGMP concentrations (fmol/mg protein cGMP) were determined (n=4) in (A) platelets and (B) plasma before (t=0) and after (t=30, 90, and 120 min) LA419 infusion. Note the trend to higher values of intraplatelet cGMP after 30 minutes starting LA419 infusion (2.5 mg/kg) which remained over baseline levels during the whole infusion period.

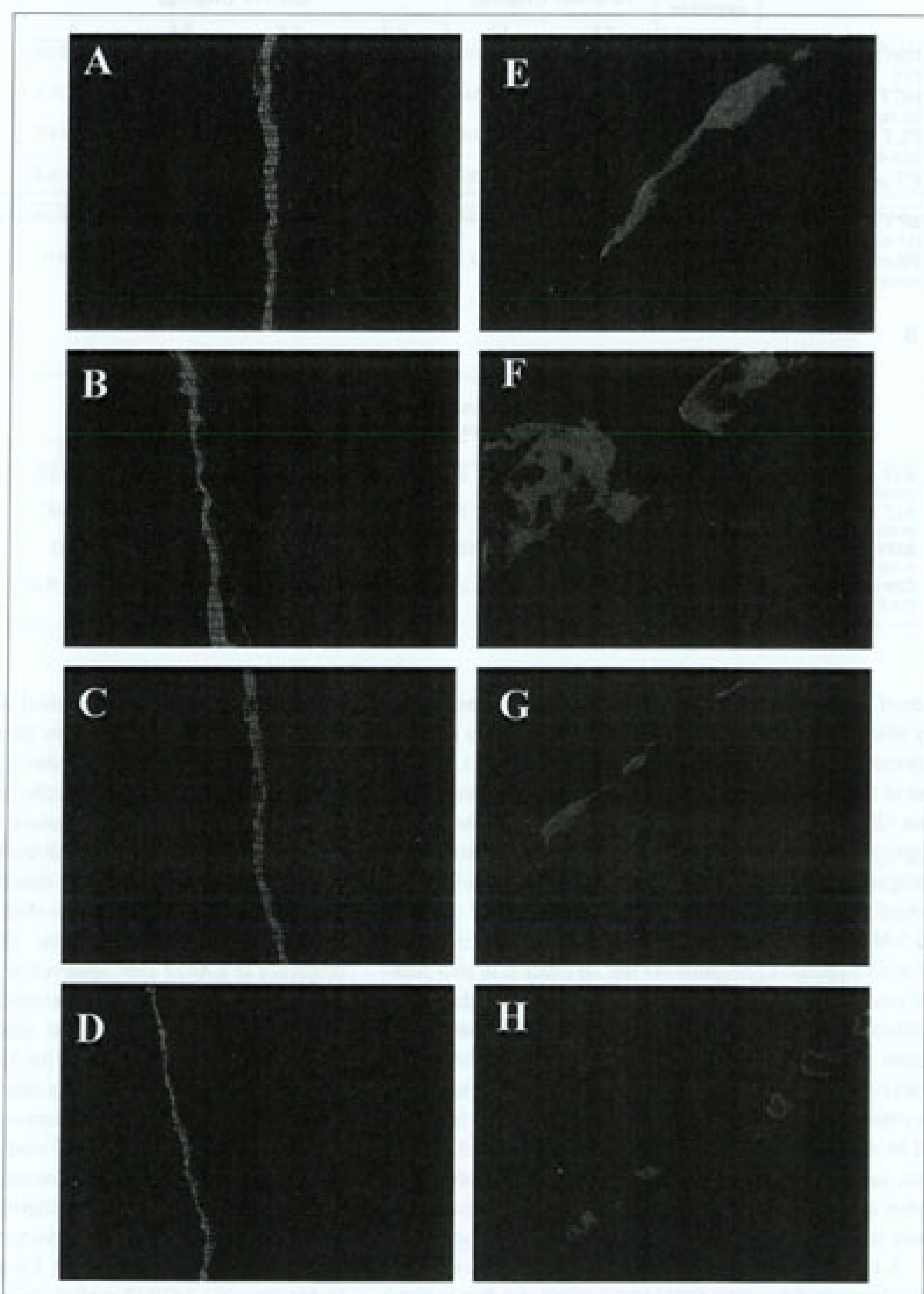


Figure 5: Representative immunophotomicrographs. Fibrin (green) (A-D) and platelet (red) (E-H) deposition on disrupted vessels at shear conditions of 1680/s in: control substrates (A, E), orally treated with IS-5-MN 0.9 mg/kg (B, F), LA419 at 1.8 mg/kg (C, G) and 5 mg/kg (D, H). No significant differences were observed on fibrin deposition while platelet deposition followed the pattern already seen in the radioisotopic quantitative analysis.

Discussion

In this study, we report that oral treatment with a new NO-donor (LA419, a neutral sugar organic nitrate with a protected thiol group in its molecular structure) (10) with tested anti-ischemic properties, decreases platelet aggregation and platelet deposition on exposed damaged vessel wall at flow conditions typical of mildly stenotic coronary arteries without modifying blood pressure. These antiplatelet effects were not observed after treatment with IS-5-MN.

The recognised role of platelets in the thrombotic processes associated to the coronary syndromes indicates that the inhibi-

A

	NORMAL AVERAGE	IS-5-MN (mg/kg)		LA 419 (mg/kg)			
		0.9	1.8	0.9	1.8	3.6	5
RBC (x10⁶/μL) (5-7)	4.9 ± 0.03	5.1 ± 0.05	4.5 ± 0.05	5.2 ± 0.04	5.2 ± 0.03	5.0 ± 0.03	5.0 ± 0.04
HCT (%) (26-35)	27.0 ± 0.1	29.0± 0.2	27.0 ± 0.2	29.4± 0.2	30.2 ± 0.2	31.4 ± 0.2	31.2 ± 0.1
PLT (x10⁵/μL) (250-450)	3.5 ± 6.0	4.0 ± 4.0	4.3 ± 13.0	3.9 ± 4.0	3.5 ± 7.0	3.5 ± 7.0	4.0 ± 14.0
PT (s)	12.8 ± 0.1	12.0 ± 0.1	12 ± 0.05	12.7 ± 0.1	12.8 ± 0.1	12.7 ± 0.1	13.7 ± 0.2
aPTT (ratio) (1.5-2.5)	2.5 ± 0.04	2.0 ± 0.06	1.5 ± 0.07	2.5 ± 0.05	1.7 ± 0.03	1.7 ± 0.04	1.6 ± 0.05
Fibrinog. (mg/dL) (170-210)	198 ± 2	187 ± 3	180 ± 2	195 ± 4	189 ± 3	186 ± 3	170 ± 6

B

	IS-5-MN (mg/kg)			LA419 (mg/kg)				
	Before treament	After 10 days treatment		Before Treatment	After 10 days treament			
		0.9	1.8		0.9	1.8	3.6	5
AST (IU/L) (15.3-55.3)	21±7	16±1	81±22*	31±6	24±8	32±11	31± 6	64 ±20*
ALT (IU/L) (9-43)	29± 2	30±3	44±1*	34±3	31±0	39±2	42±1	46±6*
BUN (mg/dL) (6-30)	8± 0.3	7± 0.8	11± 0.3	10± 0.3	8±0.4	7±0.5	9±0.5	8±1
Crea. (mg/dL) (0.5-2.1)	1.2 ± 0.1	1.2± 0.07	1.1± 0.02	1.2± 0.06	1.3± 0.05	1.1± 0.05	1± 0.05	1.1± 0.2

Table 1: Hematological, coagulation (A) and biochemical (B) parameters in non-treated and nitrate treated groups. Red blood cells (RBC); hematocrit (HCT); platelets (PLT); prothrombin time (PT); activated partial tromboplastin time (aPTT); fibrinogen (Fibrinog.). Values are expressed as mean ± standard error. *p<0.05.

tion of platelets by nitrates may offer an additional mechanism by which these compounds could improve perfusion to ischemic myocardium. Conflictive results have been found with i.v. and oral use of nitrates *in vitro* and *in vivo*, some reports showing inhibition (20-22) and others reporting no effect (23-25) on platelet aggregation and thrombosis. We have used a conventional and recognised oral nitrate, IS-5-MN, as positive control for the potential antiplatelet effect of LA419. A dose of 0.9 mg/kg of IS-5-MN, as described in the literature (26), showed inhibition of *in vitro* platelet aggregation but had no effect in *in vivo* platelet mural thrombosis. These differences seem to be due to the methodology used in platelet aggregation. Indeed, *in vitro* aggregation requires blood collection in citrate that depletes Ca^{++} from the sample and analyzes platelet response to a single agonist. In contrast, platelet deposition in the perfusion system is triggered by vascular wall, in flow conditions that mimic the circulation, and there is no Ca^{++} depletion, therefore Ca-dependent enzymes are fully available. These factors relevant in *in vivo* thrombosis are not accountable for in *in vitro* platelet aggregation.

A higher dose of IS-5-MN inhibited platelet deposition *in vivo*; however, the concomitant hepatic injury and drop in blood pressure abolishes its therapeutic use. Indeed, the therapeutic window for IS-5-MN seems not to include a platelet inhibitory effect of relevance in vascular lesion-triggered thrombosis. Interestingly Wallen et al. in a study in healthy subjects showed that isosorbide dinitrate only had platelet effects in individuals showing significant hemodynamic responses to the drug (22). In contrast, 0.9 mg/kg LA419 showed a persistent decrease in platelet-thrombus formation at low shear rate conditions, without hemodynamic effects. Thrombus inhibition at higher shear rates (a higher thrombogenic stimulus) required higher doses of LA419, but doses that did not produce a blood pressure drop.

An important limitation in nitrate therapy resides in their hemodynamic side effects (7). It should be noted that the antithrombotic effect of IS-5-MN was observed at doses that significantly decreased blood pressure. However, antithrombotic properties of LA419 were observed at doses without hypotensive episodes. Therefore, LA419 seems to have antiplatelet therapeutic efficacy at doses without hemodynamic side effects. These results suggest that LA419 has higher platelet specificity, probably due to the presence of a thiol group in LA419 structure, which facilitates nitrate conversion to NO. Thus, the hypotensive effects induced by LA419 would probably be observed, but at doses much higher than those required to achieve an antithrombotic effect. A potential deleterious effect of nitric oxide on hepatic function (27) was evidenced by higher ALT, AST serum levels after treatment with 1.8 mg/kg IS-5-MN and the higher dose of LA419 (5 mg/kg). Taken together, our findings indicate that LA419 could be a potentially useful and safe agent in the treatment of ischemic heart disease.

The proposal that Nitroglycerin may exert its activity independently of its NO-releasing properties has been done by Kleschyov et al. (28). However, this study was done *in vitro* and a proper *in vivo* study must be done before this theory can be

approved. Moreover, since the pharmacodynamic and pharmacokinetic-metabolism properties of the different NO-donors differ so much, then what could be proven for Nitroglycerin should be also proven for other NO-donors. To this regard, since we have demonstrated the maintained increase in cGMP levels after LA419 administration, and that its anti-platelet activity is avoided by a NO-scavenger (carboxy-PTIO) (data not shown), we should conclude that the anti-thrombotic activity of LA 419 should be mediated by the release of NO from that molecule.

A significant reduction on fibrinogen levels by NO has been previously reported (29) and it may be considered that if the reduction does not reach the level of hypofibrogenemia, it would diminish risk of ischemic heart disease associated to high plasma fibrinogen levels (30).

In conclusion, this study provides evidence of the *in vivo* effects of the novel compound LA419 in comparison with IS-5-MN. Oral administration of this novel NO-donor inhibits platelet mural thrombosis without modifying blood pressure. The antiplatelet effects of LA419 increase the potential benefit of this anti-ischemic molecule in patients with ischemic heart disease.

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