# *In vivo* antithrombotic synergy of oral heparin and arginine: Endothelial thromboresistance without changes in coagulation parameters

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**Summary**

On the basis of suggested clinical efficacy in an uncontrolled study in ninety-seven patients with unstable angina, an animal study was conducted to investigate antithrombotic synergy between orally administered heparin and arginine. A rat venous thrombosis model tested the difference in thrombus formation when heparin (7.5 mg/kg) and arginine (113 mg/kg) were administered, alone or in combination, by stomach tube with a minimum of 20 rats/group. Oral heparin, arginine, and heparin plus arginine reduced thrombus formation by 50%, 75%, and 90%, respectively, when compared to saline administration. Heparin was recovered from endothelium, yet there was little or no observable plasma anticoagulant activity. An orally administered low-molecular-weight anticoagulant glycosaminoglycan mixture, sulodexide (7.5 mg/kg), showed an 88% reduction in stable thrombus formation when administered alone but showed no synergy with oral arginine. A 28-day study with oral sulodexide (2.9 mg/kg) and arginine (43.9 mg/kg), 20 rats/group, showed antithrombotic activity with minimal anticoagulant activity indicating suitability for long term treatment. These findings suggest the endothelial localization of heparin and a synergistic antithrombotic effect for orally administered heparin and arginine.

**Keywords**

Heparin, thrombosis, endothelium, arginine, oral

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## Introduction

Previous investigations have demonstrated that heparin has antithrombotic activity when administered orally in rat thrombosis models (1, 2). Several studies suggest that heparins are found on the endothelial surface within minutes following oral administration with minimal observed evidence of plasma distribution. Endothelial concentrations of heparin are reported to be 1,000 –10,000 times the levels measurable in plasma following oral administration to rats (3, 4). If similar effects occur on human endothelium following oral heparin administration, potent antithrombotic effects would be anticipated without systemic anticoagulation.

Arginine and heparan sulfate have important roles in the functional and molecular organization of vascular homeostasis and hemostasis. Arginine is the substrate for eNOS in the endothelial production of NO (5). NO reduces endothelial adhesiveness, prevents platelet aggregation, and results in vasorelaxation (6–8). Arginine is also reported to have anticoagulant properties (9). Arginine groups on peptides are major determinants in their interaction with endogenous heparan sulfates on the endothelial surface of blood vessels (10). While arginine only weakly interacts with the *O*-sulfoesters of heparan sulfate and heparin, this binding is stronger than that of other basic amino acids (11). Furthermore, heparin induces eNOS activity (12), and heparin as well as NO increase the biosynthesis of endothelial heparan sulfate with increased amounts of ATIII binding sites (13, 14). Evidence also suggests both NO and heparan sulfate deficiencies may cause endothelial dysfunction, prothrombotic effects, and plaque formation (15–21). Thus, an important relationship exists between heparin, heparan sulfate, arginine and NO in promoting antithrombotic activity and vascular

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health. Based on these reports, we speculated that administration of both heparin and arginine together would simultaneously increase endothelial heparan sulfate and NO and possibly exhibit antithrombotic synergy. Since oral administration of heparin in rats results in its enhanced deposition on the endothelium relative to plasma levels (3), we chose this route for our investigation.

We report here beneficial cardiovascular effects in a series of patients, with unstable angina in a clinical setting, treated for 12 months with oral heparin plus arginine. Data are also presented confirming synergy of oral heparin plus arginine in inhibiting thrombus formation in an established rat venous thrombosis model of 4-hour duration. Oral sulodexide, a glycosaminoglycan mixture (22), had antithrombotic activity in a 4-hour and 28-day study when combined with arginine.

## Materials and methods

### Patients

Ninety-seven consecutive patients diagnosed with unstable angina were treated with the combination of 10,000 units of standard porcine intestinal heparin, sodium USP delivered orally in 125 – 200 ml of water, combined with arginine (Jo Mar Laboratories, Campbell, CA, USA) administered as two 825 mg capsules by mouth twice daily. The total L-arginine dose was 3.3 grams daily in all patients. The patients were instructed to immediately go to the emergency room for admission if their symptoms recurred or accelerated during the subsequent 48 hours. Patients were referred for interventional treatment if symptoms failed to resolve within 72 hours and if symptoms recurred at any time within 12 months. All other current medications were continued, patients remained on aspirin, beta blockers, statin drugs and other treatments as were prescribed prior to the diagnosis. The resulting combined cardiac event rates were compared to a series of patient outcomes at 12 months post diagnosis of unstable angina as reported in the recent FRISC II Trial (23). This uncontrolled observational study was conducted in a private medical practice. Since this was not a controlled trial, no informed consent was obtained.

### Animal studies

#### Drugs

Unfractionated bovine lung heparin (sodium heparin 150 units/mg, Lot No ZX320, Upjohn Ltd., Kalamazoo, MI, USA) was dissolved in water at a concentration of 20 mg/ml. Sulodexide, a glycosaminoglycan mixture containing fast moving heparin 80% and dermatan sulfate 20% (22), was obtained from Keryx Biopharmaceuticals (New York, NY, USA). Sulodexide was dissolved in water at a concentration of 20 mg/ml for short-term studies and 10 mg/ml for long-term studies. Gelatin capsules containing arginine, similar to those used in the human studies, were opened, and contents were dissolved in water at a concentration of 300 mg/ml for 4-hour studies and 150 mg/ml for the 28-day study.

#### Animals

One hundred eighty-two male Wistar rats, weighing 311 ± 48 g (± SD), were handled and housed according to the Principles of Animal Care set out by the Canadian Federation of Biological Societies. The animals were fasted overnight prior to treatment and were anaesthetized with barbital and methoxyflurane for experimental procedures.

#### Drug administration

For short term studies the following treatment groups were used: saline, heparin, arginine, heparin plus arginine, sulodexide, and sulodexide plus arginine. Bovine heparin was used in the animal studies in place of the porcine heparin used in the human study. The antithrombotic response is similar for the two heparins when given orally in the rat venous thrombosis model (26). Heparin and sulodexide were administered at 7.5 mg/kg based on previous studies showing a 50% decrease in stable thrombus formations with unfractionated heparin in this animal model (2). Arginine was administered at 112.5 mg/kg to approximate the dose used in the human study. The dose of heparin or sulodexide was assigned randomly with 6 to 8 rats treated per day. A stomach tube was filled with 0.2 ml saline followed by 0.10 – 0.18 ml of heparin or sulodexide solution and 0.1 ml of arginine solution depending on rat weight. Thus, when the stomach tube was placed in the stomach, the drugs were first introduced into the stomach and flushed in by saline to give a total volume of approximately 0.4 ml. In the heparin only group, heparin was administered in a volume of 0.1 – 0.2 ml followed by 0.2 ml saline. For long-term studies sulodexide (2.9 mg/kg) plus arginine (42.9 mg/kg) were given daily by stomach tube for 28 days. Control rats treated for four hours were given saline (0.3 ml) by stomach tube. Control rats treated for 28 days were kept in metabolic cages during that time and were given water.

#### Thrombosis test

The thrombosis test was performed in a modification of the procedure by Blake et al. (24). For animals exposed to treatment for 4 hours, a thrombus was initiated in the right jugular vein by application of 10% formalin in 65% methanol to the exposed adventitial surface. Immediately following, drugs were introduced into the stomach by stomach tube. At four hours after thrombus initiation, animals were again deeply anaesthetized and first examined for any external signs of bleeding. The jugular vein was examined for the presence of a plug using a cotton pledget. The thrombus was scored as + (stable thrombus) if the vessel was blocked and remained blocked despite examination with a cotton pledget. The thrombus was scored as +/- (soft or unstable thrombus) if the vessel appeared completely blocked on first examination and then opened as it was examined. The thrombus was scored as – (negative) if blood was seen to flow freely in the vessel.

For the long-term (28-day) study, a thrombus was initiated in the jugular vein on the final day, four hours prior to killing the rats. The last ($28^{th}$) dose was administered immediately after thrombus initiation. The thrombus was evaluated as described for short-term studies.

#### Collection of blood and blood vessels

Immediately after examination of the jugular vein, a laparotomy was performed and a blood sample of approximately 10 ml (9 parts blood to 1 part 3.8% sodium citrate) was taken from the abdominal aorta. Plasma was prepared. As a source of endothe-

lium, the thoracic aorta was removed and placed in saline. Each animal was examined for signs of internal hemorrhage, and the time when blood clotted in the body cavity was recorded.

### Harvesting of endothelium

Endothelium was removed from blood vessels according to the method of Hiebert and Jaques (25). The vessels were slit open, pinned to dental wax lumen side up, and rinsed in Locke's solution. Cellulose acetate paper was applied to the luminal surface and when lifted, endothelium was removed. The length and width of the imprint were measured to the nearest millimeter. Harvested endothelial surface was 2.28 ± 0.74 (SD) and 0.57 ± 0.25 $cm^2$ for aorta and vena cava, respectively.

### Determination of heparin and sulodexide with endothelium

Cellulose acetate paper was removed from endothelium by dissolving in cold acetone followed by centrifuging and discarding the supernatant. The precipitates were then digested with pronase (Sigma, St. Louis, MO, USA; 10 µg of 40 mg/ml in Tris buffer). Samples were then centrifuged at 8,000 x g for 10 min, supernatant was collected and the precipitate washed twice with 100 µl 26.8% NaCl that was added to the supernatant. GAGs were precipitated from the supernatant with five volumes of methanol, and the precipitate was dried. Agarose gel electrophoresis was used to identify and measure heparin in endothelial extracts by previously published methods (1). The dried powders were dissolved in water and applied to agarose gel slides along with the heparin or sulodexide used as references. Following electrophoresis, gels were fixed in 0.1% hexadecyltrimethylammonium bromide and air-dried. Slides were stained with 0.04% toluidine blue in 80% acetone, and background color was removed with 1% acetic acid. Heparin and sulodexide were identified by comparing migration to reference material. Amounts contained in each sample were determined by densitometry.

### Determination of plasma anticoagulant activity

The activated partial thromboplastin time (APTT) was determined using a kit from Biopool (Ventura, CA, USA). Anti-factor Xa and anti-factor IIa activity were measured using chromogenic assay kits, Accucolor ™Heparin ® (Sigma) and Spectrolyse ® Heparin, respectively.

Heparin concentration in plasma was also measured using the Heptest (Accuclot ™ Heptest®, Sigma) where the clotting time determined was converted to µg/ml by use of calibration curves prepared at the same time with unfractionated bovine lung heparin or sulodexide.

### Determination of heparin and sulodexide in urine

Rats were placed in metabolic cages, and urine was collected over the 4-hour period, or 24-hour urines were collected on day 1, 7, 14, 21 and 28 in the 28-day study. Urine was dialyzed against water using 1,000 molecular weight cut off (MWCO) dialysis tubing (Spectrum Chemical and Laboratory Products, Gardena, CA, USA). The resulting solution was dried and analyzed by agarose gel electrophoresis as described above for endothelium.

### Statistical analysis

Thrombosis data is expressed as a percentage with 95% confidence intervals (2). Test for differences between proportions was used to compare the total thrombotic incidence and incidence of hard thrombus between groups. Rat weight and surface area of endothelium are expressed as mean ± standard deviation (SD). Other data is expressed as mean ± SE. A one-way ANOVA with Tukey's post hoc test was used to compare the differences between groups when plasma coagulation tests and heparin or sulodexide concentrations in urine were examined.

## Results

### Patients

Results from the cohort of patients on daily maintenance of heparin plus arginine, 12 months post diagnoses, are shown in Table 1. These data show that the cardiac event rates (death, myocardial infarction, readmission and revascularization) were dramatically reduced compared to published data using other treatment options. No other complications were observed in any patient. These results compare favorably to those of the recent FRISC II trial (23) at 12 months and show a dramatic reduction of 49 – 93% seen in death, myocardial infarction or secondary cardiovascular events without use of interventional treatment and a reduction of 9 – 89% with interventional treatment.

### Animal studies

On the basis of these anecdotal results, showing effectiveness of oral heparin and arginine on prothrombotic activity in humans, an animal model was used to investigate possible mechanisms. The rat jugular vein model was chosen as it is well established and has been previously used to study the antithrombotic activity of heparins given by the oral route (2). Since most of the previous studies in this venous thrombosis model have been conducted using bovine lung heparin, this source was chosen to study its effects with arginine. Sulodexide, a glycosaminoglycan mixture was also chosen in the repeated dose long-term study of 28 days, since it reportedly has lower anticoagulant activity and is suitable for long-term oral administration (22). We measured antithrom-

**Table 1: Combined cardiovascular event rates with oral heparin and arginine as compared to FRISC II trial (subcutaneous fragmin treated patients).**

| | Oral heparin/ arginine (n = 97) | Non-invasive FRISC II group (n = 1234) | Difference (reduction) | Invasive* FRISC II group (n = 1222) | Difference (reduction) |
|---|---|---|---|---|---|
| Death | 2 (2.0%) | 48 (3.9%) | 49%, P=0.58† | 27 (2.2%) | 9%, P=1 |
| Myocardial infarction | 3 (3.0%) | 143 (11.6%) | 75%, P= 0.006 | 105 (8.6%) | 65%, P=0.055 |
| Readmission | 4 (4.1%) | 704 (57.1%) | 93%, P< 0.0001 | 451 (36.9%) | 93%, P< 0.0001 |
| Revascularization | 3 (3.0%) | 383 (31.0%) | 90%, P< 0.0001 | 92 (7.5%) | 90%, P=0.15 |

*The principal goal of the Fragmin and fast revascularization during instability in coronary artery disease II (FRISCII) study was to compare the effectiveness of invasive vs. non-invasive treatment in terms of the incidence of death and myocardial infarction in patients with unstable coronary artery disease (CAD). The results indicated that an early invasive approach is preferable in the treatment of patients with unstable CAD. Invasive procedures were revascularization by percutaneous transluminal coronary angioplasty or bypass grafting.†Fisher's exact test.

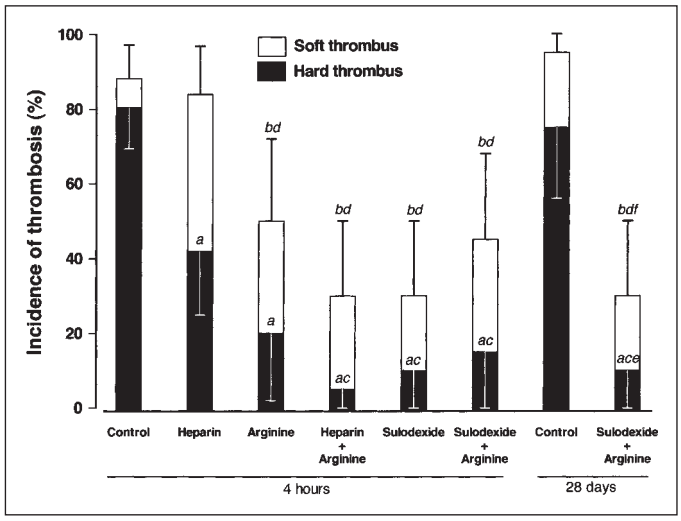


**Figure 1: Thrombosis incidence following administration of heparin, sulodexide and/or arginine in a rat model.** Thrombosis was initiated by application of formalin in methanol to the exposed right jugular vein. In the 4-hour studies, heparin (7.5 mg/kg) or sulodexide (7.5 mg/kg) and/or arginine (112.5 mg/kg) were administered, immediately after thrombus initiation, by stomach tube. Incidence of thrombosis was evaluated four hours later. In the 28-day study, sulodexide (2.9 mg/kg) and arginine (42.9 mg/kg) were administered daily by stomach tube. A thrombus was initiated four hours prior to the end of the experiment. Upward deflecting error bars show 95% confidence intervals for total thrombotic events, downward deflecting bars show 95% confidence intervals for hard thrombi. Significantly less than: 4-hour control incidence of hard thrombi (A); 4-hour control total thrombotic incidence (B); heparin alone incidence of hard thrombi (C); heparin alone total thrombotic incidence (D); 28-day control incidence of hard thrombi (E); 28-day total thrombotic incidence (F). ($Chi^2$-test for goodness of fit.).

botic activity, compounds recovered from the endothelium and urine, and anticoagulant activity following administration of heparin and sulodexide with and without arginine.

### Thrombosis incidence

In the 4-hour studies, rats treated with saline (control) showed an 80% incidence of hard thrombi and an 88% incidence of total thrombotic events (hard plus soft thrombi) (Fig. 1). When oral heparin (7.5 mg/kg) was administered, the incidence of hard thrombi was significantly reduced to 42% although the total thrombotic incidence was minimally affected. When arginine (112.5 mg/kg) was administered alone, the incidence of hard thrombi was reduced to 20%, significantly different from control but not from heparin treated rats, while total thrombotic events were 50%, significantly less than both control and heparin treated rats. When arginine plus heparin (7.5 mg/kg) were administered orally, the incidence of hard thrombi was reduced to 5% and the total thrombotic events were reduced to 30%, both significantly less than control or heparin treated rats. When using sulodexide (7.5 mg/kg) alone in the 4-hour study, the incidence of hard thrombi was 10% and the total thrombotic incidence was 30%, both significantly less than observed for control or heparin treated rats. When arginine plus sulodexide were administered, the incidence of hard thrombi was 15%, and total thrombotic incidence was 45%, both significantly less than control or heparin treated rats but not different than sulodexide treatment alone.

In the long-term study, control rats kept in metabolic cages for 28 days showed a 75% incidence of hard thrombi and a 95% incidence of total thrombotic events. When rats were treated with sulodexide (2.9 mg/kg) plus arginine (42.9 mg/kg) for 28 days, incidence of hard thrombi (10%) and total thrombotic events (30%) were similar to rats treated four hours with sulodexide alone or with sulodexide (7.5 mg/kg) plus arginine (112.5 mg/kg). Rats treated for 28 days with sulodexide plus arginine showed significantly less thrombotic events than 4-hour and 28-day treated control rats, and 4-hour heparin (7.5 mg/kg) treated rats.

### Recovery from endothelium

Extracts of endothelium applied to agarose gel electrophoresis showed that heparin and sulodexide were detected in endothelial samples, as determined by both their migration distance and their staining characteristics (Fig. 2). The concentrations of heparin and sulodexide found with endothelium are shown in Table 2. As expected, endogenous glycosaminoglycans, migrating similar to sulodexide, were observed in control endothelial samples while little if any endogenous heparin was detected in these samples. The endothelium from rats treated with oral heparin clearly showed the presence of heparin. Concentrations of heparin in both the aortic and vena caval endothelium of rats were greatest when treated with heparin alone, followed by those treated with heparin plus arginine and then by controls. The mean concentrations of heparin with endothelium of rats treated with heparin alone were more than an order of magnitude greater than that found with endothelium of control rats, while endothelium from rats treated with heparin plus arginine were four to five times greater than control endothelium. In general, more heparin and sulodexide were recovered from vena caval endothelium than from aortic endothelium.

The recovery of sulodexide from the vena caval endothelium was greatest for rats treated for 28 days with sulodexide plus arginine. Sulodexide concentrations on aortic endothelium, for sulodexide plus arginine treated groups, was more than twice that

**Table 2: Sulodexide or heparin with endothelium following administration with and without arginine.**

| Treatment | Duration of treatment | Aortic concentration µg/cm² | Vena Caval concentration µg/cm² |
|---|---|---|---|
| Control | 4 h | 0.02 ± 0.007 | 0.05 ± 0.03 |
| Control** | 4 h | 0.04 ± 0.01 | 0.05 ± 0.04 |
| Heparin | 4 h | 0.28 ± 0.13 | 1.27 ± 0.49 |
| Arginine** | 4 h | 0.04 ± 0.01 | 0.11 ± 0.05 |
| Heparin + Arginine | 4 h | 0.08 ± 0.02 | 0.25 ± 0.14 |
| Sulodexide** | 4 h | 0.03 ± 0.01 | 0.15 ± 0.08 |
| Sulodexide+ Arginine** | 4 h | 0.09 ± 0.03 | 0.12 ± 0.03 |
| Sulodexide+ Arginine*** | 28 day | 0.06 ± 0.01 | 0.21 ± 0.04 |

* Doses for the 28 day study were sulodexide 2.9 mg/kg/day; arginine 42.9 mg/kg/day. All other groups were given single doses of heparin or sulodexide at 7.5 mg/kg; arginine 112.5 mg/kg.
** compared to sulodexide standards, all other groups compared to heparin standards.

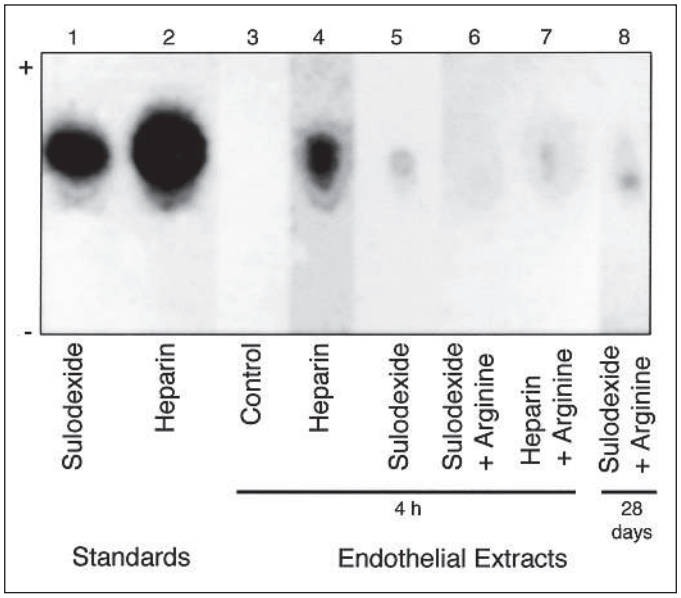


**Figure 2: Agarose gel electrophoresis slides showing heparin and sulodexide with endothelium following oral administration.** Lane 1, sulodexide standard; lane 2, heparin standard; lane 3, rat endothelium extract in the absence of administered agent (control); lane 4, rat endothelium extract four hours after 7.5 mg/kg oral heparin; lane 5, rat endothelium extract four hours after 7.5 mg/kg oral sulodexide; lane 6, rat endothelium extract four hours after 7.5 mg/kg oral sulodexide + arginine 112.5 mg/kg; lane 7, rat endothelium extract 4 hours after 7.5 mg/kg oral heparin + arginine; and lane 8, rat endothelium extract 28 days after 2.9 mg/kg/day oral sulodexide + arginine 42.9 mg/kg/day.

of the control group. Endothelium from rats treated with arginine alone showed twice the concentrations of sulodexide on aortic and vena caval endothelium as that from control rats.

### Anticoagulant activity

APTT, Heptest and anti-factor Xa activity was assessed to determine if sulodexide or heparin and/or arginine modified coagulation following oral administration (Fig. 3). Although only small changes were observed in APTT in the 28-day study of the sulodexide plus arginine group, the APTT in the 28-day group was significantly greater than the 4-hour treated sulodexide plus arginine, the heparin plus arginine or the arginine alone treated groups. Interestingly, anti- factor Xa activity was significantly greater in controls than in the groups treated with heparin plus arginine and arginine alone. In addition, the 28-day sulodexide plus arginine treated group had noticeably lower anti-factor Xa activity, and this activity was significantly lower than the heparin plus arginine treated, sulodexide treated and arginine alone treated groups.

Little or no anticoagulant was found in plasma as determined by the anti-IIa assay. No anti-IIa activity was detected in plasma of the 4-hour sulodexide plus arginine, heparin plus arginine and the 28-day long-term sulodexide plus arginine groups. A small amount of anti-IIa activity was determined in the plasma of animals administered sulodexide alone and arginine alone (values equivalent to 1.43 ± 0.97, and 4.36 ± 5.04 [SE] g/ml, respectively).

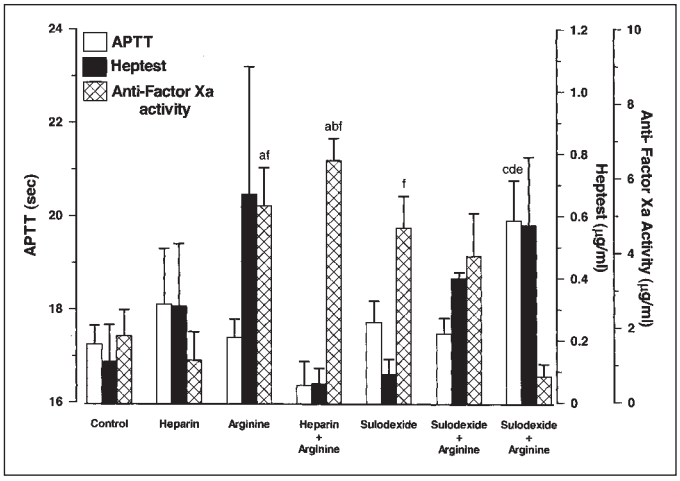


**Figure 3: Changes in coagulation parameters following administration of sulodexide or heparin plus arginine to rats.** In the 4-hour groups, heparin (7.5 mg/kg) or sulodexide (7.5 mg/kg) and/or arginine (112.5 mg/kg) were administered by stomach tube four hours prior to blood collection. In the 28-day study, sulodexide (2.9 mg/kg) and arginine (42.9 mg/kg) were administered daily by stomach tube, the last dose given four hours prior to blood collection. One-way ANOVA, Tukey's post hoc test. Results are significantly greater than control (a); heparin (b); heparin plus arginine (c); sulodexide plus arginine (d); arginine (e); sulodexide plus arginine 28 days (f). P <0.05.

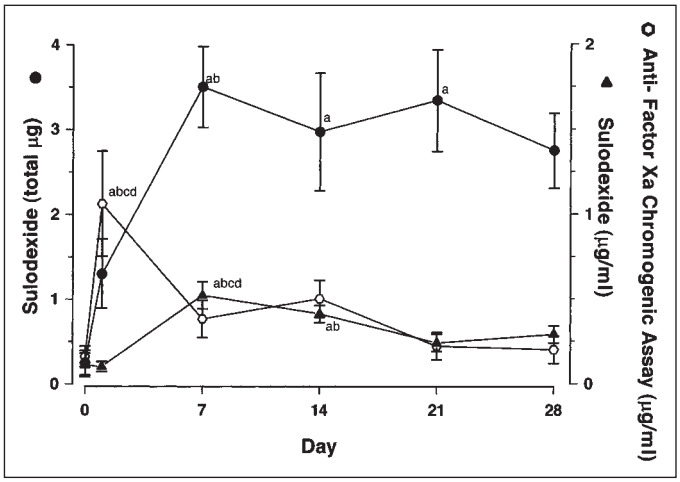


**Figure 4: Sulodexide-like compounds in 24-hour urine samples at different days following long-term administration of sulodexide plus arginine.** Sulodexide 2.9 mg/kg plus arginine 42.9 mg/kg were given by stomach tube daily for 28 days. Mean ± SE are shown. One-way ANOVA. Tukey's post hoc test; significantly greater than control (a); day 1 (b); day 21 (c); day 28 (d). P < 0.05.

### Recovery from urine

When changes in the amount and concentration of urinary sulodexide with time were determined by electrophoresis with time for animals administered sulodexide plus arginine for 28 days, maximum increases in total µg and µg/ml were seen at day 7 (Fig. 4). While the total amount of sulodexide remained elevated throughout the 28-day period, its concentration in the urine declined after day 7. With the exception of a large spike on day 1,

urinary anti- factor Xa activity levels closely paralleled the concentrations found by chemical determination. No anti-factor IIa activity was found in urine samples from control or experimental animals. Heparin and sulodexide were found only in small amounts in the urine of rats treated for four hours with heparin or sulodexide, with heparin only treated animals showing levels significantly greater than control rats (data not shown).

## Discussion

The results of the study of oral administration of heparin plus arginine in unstable angina patients, suggest that this therapeutic approach compares favorably to both strategies in patients receiving subcutaneous LMWH in both invasively and non-invasively treated groups (23). Controlled human studies, using a larger number of patients, are required to establish the significance of heparin plus arginine on the various endpoints, particularly patient death. Based on these anecdotal results, the same combination dosage of oral heparin plus arginine, as well as oral sulodexide plus arginine, and each agent alone were investigated in a rat jugular vein thrombosis model. The results of this animal study clearly demonstrate that orally administered arginine has an antithrombotic effect in this rat thrombosis model as does orally administered heparin. In addition, the administration of heparin plus arginine has additional antithrombotic effects when compared to heparin or arginine alone. Arginine did not have the same synergistic effect when combined with sulodexide, although sulodexide alone had antithrombotic activity. These results suggest that sulodexide and arginine may act by a similar mechanism whereas heparin has additional antithrombotic effects. Sulodexide alone was more effective than heparin alone in the short-term study. This may be due to the presence of dermatan sulfate or possible shorter chain length heparin (Mr (avg) ~ 8,000) present in this glycosaminoglycan mixture (22). These components of sulodexide may exhibit a faster rate of oral absorption and slower rate of clearance than observed with the unfractionated heparin (Mr (avg) ~ 12,000) used here. This is consistent with our previous results in which low molecular weight heparins were effective at much lower doses than unfractionated heparins (2).

A decision to do the long-term, 28-day study with only sulodexide and arginine was based on the assumption that sulodexide would cause less bleeding in long-term studies because of its reduced anticoagulant activity compared to heparin. It is interesting to note that 28-day administration of sulodexide and arginine was effective in reducing thrombosis incidence but not significantly different than 4-hour administration. However, the dose administered per day over the 28-day period, was less than in the 4-hour study. This suggests that long-term administration results in an accumulation of the drug or its beneficial effects. Further studies are warranted to examine this phenomenon, perhaps beginning with using the lower dose in a short-term experiment (4 hours).

As in previous experiments (1–3, 26), heparin was recovered from aortic and vena caval endothelium following its oral administration. In the present studies, arginine alone appears to increase the concentration of endogenous glycosaminoglycans on the vena caval endothelium, consistent with the studies indicating that arginine upregulates the heparan sulfate on the endothelial cell surface. The highest recovery from endothelium was obtained in the group treated with heparin alone. Moreover, the vena caval endothelium generally showed substantially higher concentrations of heparin and glycosaminoglycan than the aortic endothelium, agreeing with our previous work on orally administered low-molecular-weight heparins (4, 27). Heparin, however, was not recovered from the endothelium of all animals that showed antithrombotic activity. This is probably the result of both the limited sensitivity of our assay and the sampling of only a small amount of the total endothelium, an average of 2.9 $cm^2$ of approximately 2 $m^2$ of total endothelial surface. Since the migration of sulodexide under electrophoresis is similar to endogenous endothelial glycosaminoglycans, it was difficult to establish the presence of the exogenously administered sulodexide on the endothelium.

No evidence of gastrointestinal irritation or bleeding was observed in either humans or rats during these investigations. There were only minor changes in the plasma anticoagulant activity following oral administration of sulodexide or heparin as compared to controls. Noteworthy is the observed increase in APTT in the group receiving long-term sulodexide plus arginine. A similar small but significant increase in APTT was seen in humans receiving repeated oral heparin at 20,000 units twice weekly for two to eight months (28). Our own work with dextran sulfate showed a similar increase in APTT in humans treated 29–335 days with 1 gram four times per day of dextran sulfate. This occurred despite dextran sulfate having one hundreth the anticoagulant activity of heparin (29). In the present study, anti-factor Xa activity was significantly elevated in the arginine alone, and the heparin plus arginine treated groups, leading one to speculate that arginine may have some effect on anti-factor Xa activity. In contrast, anti-factor Xa activity was low in the long-term 28-day arginine plus sulodexide treated rats. A possible explanation for this low anti-factor Xa activity is that long-term administration of sulodexide and arginine may result in changes in production or consumption of coagulation factors to balance the increasing anticoagulant effect of sulodexide or arginine.

Consistent with heparin binding to endothelium, little heparin or sulodexide was recovered from urine in the 4-hour study with only the group receiving heparin alone showing a significant increase in urinary heparin concentration compared to controls. This result might be explained by a faster rate of absorption or excretion for heparin than for sulodexide, or for heparin or sulodexide in combination with arginine. Small amounts of heparin or sulodexide were found in the urine of all rats, as expected. In contrast to 4-hour administration, 28-day administration of sulodexide and arginine showed significant increases in glycosaminoglycans in the urine, beginning on day 1 when anti-factor Xa activity was measured, and on day 7 when measured chemically. This supports the observations that sulodexide is absorbed, and that some accumulation occurs throughout the 28-day period, resulting in an increase in excreted material.

Since this study shows that oral arginine plus heparin has significantly increased antithrombotic activity compared to oral heparin alone, we speculate that arginine enhances the absorption of heparin, and/or arginine enhances heparin's antithrombotic activity. This is supported by the observation that anti-factor Xa

activity is significantly higher in heparin plus arginine treated rats than in heparin only treated rats. (It should be noted that these changes in anti-factor Xa activity are minor although significant, Fig. 3). Results from the analysis of the endothelium in this study also show less heparin on the endothelium in the presence of arginine than when heparin is given alone.

The clinical results reported suggest that heparin is absorbed in humans following oral administration and is distributed primarily to endothelium. These studies are supported by the work of Engelberg who showed increases in APTT in a human study with oral heparin (28) and by our own studies showing an increase in anti-factor Xa activity and heparin in urine following a single dose of 7.5 mg/kg of heparin to human subjects (30). A recent study also confirms similar endothelial deposition of heparin as well as antithrombotic efficacy when administered orally in a rat model of carotid artery thrombosis (31). In the current study, the observation that arginine enhances the activity of orally administered heparin in both humans and animals, despite little change in plasma anticoagulant activity, is a new finding and suggests that arginine and heparin have a synergistic antithrombotic effect primarily on the endothelium.

The exact mechanism by which arginine enhances the antithrombotic effect of oral heparin remains unknown, although enhanced absorption or enhanced endothelial antithrombotic activity, may contribute to these effects. Arginine only weakly binds heparin (11), so that it is unlikely that arginine interacts with heparin in the body due to the presence of salts and competing, higher-affinity heparin-binding proteins. We suggest instead that it is likely that heparin and arginine exert their antithrombotic through different pathways but in a synergistic fashion. Since arginine is a substrate for NO (5), these results suggest that NO may play a role in this phenomenon. Heparin increases NO activity through effects on eNOS (32). NO enhances the production of endogenous endothelial anticoagulant heparan sulfate (14). Such endogenous heparan sulfates have been shown to mediate flow-induced eNOS signal transduction (33). The cellular uptake of heparan sulfate proteoglycans has also been shown to be related to NO catabolism (20). There has also been a recent report implicating oral heparin in enhanced NO production, which accelerates healing of gastric ulcers in animals (34). Decreased excretion of heparin in the presence of arginine has not been excluded as an operative mechanism for their synergistic antithrombotic effect. Moreover, it is interesting that oral sulodexide also has antithrombotic activity that is not enhanced by its co-administration with arginine.

Both endothelial heparan sulfate and NO effects are known to be reduced in the atherothrombotic processes as well as in aging in humans (35, 36). Cholesterol plaque accumulation occurs at sites of reduced eNOS activity. Rupture sites of culprit plaque lesions, responsible for unstable angina and myocardial infarction, show markedly depleted levels of endogenous proteoglycans compared to normal artery segments (37). The marked decrease in expected cardiovascular events rates seen in such patients over a 12-month period of using daily, continuous oral heparin and arginine treatment suggests sustained passivation of plaque surfaces in these treated patients. Further studies are required to assess the roles of NO production, endogenous endothelial heparan sulfate effects, coagulation factor complex assembly, thrombolytic processes, and other questions raised by the observations reported in the current study. These results suggest the suitability of oral heparin or related glycosaminoglycans with arginine, with no demonstrated adverse reactions and absence of systemic anticoagulation effects, for long-term antithrombotic prophylaxis and treatment in larger human and animal trials.

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