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EXPERIMENTAL STUDIES ON THE ANTICOAGULANT AND ANTITHROMBOTIC EFFECTS OF SODIUM AND CALCIUM PENTOSAN POLYSULPHATE

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In the present study we have compared the antithrombotic and anticoagulant properties of sodium and calcium derivatives of pentosan polysulphate (Na-PPS, Ca-PPS).
The antithrombotic effect of these agents have been investigated in an experimental thrombosis model in which rat mesenteric venules diameter of 20—30 μm were injured by well defined Argon laser lesions. Furthemore, the *in vivo* and *in vitro* anticoagulant activities (aPTT, Heptest) of these agents have been studied.
Thrombus formation was significantly inhibited after s.c. injection of Na-PPS and Ca-PPS in doses above 10 mg/kg. The duration of the antithrombotic effect lasted 8 h for Na-PPS and 12 h for Ca-PPS. After oral administration of Na-PPS an antithrombotic effect was not observed. Oral application of Ca-PPS in doses higher than 20 mg/kg significantly inhibited thrombus formation. Na-PPS and Ca-PPS markedly prolonged clotting time in aPTT and Heptest in concentrations ranging from 0.01 to 0.2 mg/ml rat PTT. Two h after s.c. administration of these agents in a dose 10 mg/kg, the aPTT increased 3-fold and Heptest 2.5-fold compared to controls. After oral application of 50 mg/kg Na-PPS and Ca-PPS no effect on coagulation test could be measured.

Key words: *sodium and calcium pentosan polysulphate, anticoagulant activity, laser animal model, microthrombus formation.*

INTRODUCTION

Antithrombotic drugs generally used in the prophylaxis of thromboembolic complications in patients who have undergone surgery produce their clinical effects through different mechanisms which target componets of hemostasis.

Sulphated polysaccharides represent a group of drugs with various anti-thrombin and anti-Xa ratios and marked differences exist in their biological and pharmacological characteristics.

Pentosan polysulphate is a mixture of polysaccharides that is obtained by sulphation of pentosan (1, 2). Soria *et al.* (3) have reported that pentosan polysulphate (PPS) shows some similarities with low molecular weight heparin as to its inhibitory effect on blood coagulation. Low doses of PPS have already been found to lead to suppression of factor Xa generation independent of antithrombin III. At high doses the inhibition of thrombin and factor Xa was potentiated (4).

Pentosan polysulphate also is a good activator of heparin cofactor II. Dunn *et al.* (5) have shown that PPS increases the binding of fibrinogen to ADP-treated platelets. Pentosan polysulphate may enhance the fibrinolytic activity. The fibrinolytic activity induced by PPS when administered *in vivo* is due to the release of tissue-type activator (6). However, activity after oral application is still a matter of discussion.

To obtain more detailed information about the antithrombotic effects of sodium and calcium pentosan polysulphate (Na-PPS, Ca-PPS), these agents were investigated in model of experimental laser-induces venous thrombosis in rat mesenteric venules. Animal thrombosis models can be useful tools to study correlations between antithrombotic effects and the inhibition of different coagulation factors (7).

The aim of our study was to compare the antithrombotic and anticoagulant efficacy of Na-PPS and Ca-PPS after s.c. and oral administration. The *in vivo* and *in vitro* anticoagulant properties of these polysaccharides in rat platelet-poor plasma have been studied.

MATERIAL AND METHODS

Drugs tested: sodium and calcium Pentosan Polysulphate (bene-Arzeneimittel GmbH, München) was generously provided by Dr. M.E. Scholz.

Blood Sampling:

1 vol of 3.13% citrate + 9 vol of blood from abdominal aorta of rats were prepared.

Coagulation Tests: the Heptest (Haemchem, USA), and partial thromboplastin time (aPTT) using an IL 300 centrifugal analyzer (Instrumentation Laboratory, Ascoli Piceno, Italy) were applied. Blood samples were taken from rats after subcutaneous injection and oral application of polysaccharides immediately following the laser experiment which corresponded to 120—140 min after the administration. Citrated blood samples were centrifuged for 20 min at 3,000 g. Citrated plasma was frozen at −75°C, until the coagulation test were performed.

Animal experiments

Laser thrombosis model in animals: the investigations were carried out on male Wistar rats weighing 200 to 300 mg; the rats were anaesthetized with pentobarbital sodium (Nembutal ®) 60 mg/kg i.p. An intestinal loop, exposed through a hypogastric incision and continuously irrigated with saline, was spread flat on a self constructed object stage, which was mounted on the microscope table of a Leitz Orthoplan microscope equipped with interference contrast objectives (50 × 1.00 numerical aperture) and condensors. One of two oculare (× 10) contained a grating to

faciliate correct aiming of the laser lesions. The vascular lesions were induced by a Coherent Innova 70 (Argon Laser) with an effective energy of 0.09 W, which was measured below the objective of the microscope. The exposure time to produce the laser lesions was kept at 1/30 s. The laser beam was directed through the objective on the center of small mesenteric venules measuring 20—30 μm in diameter in the fat-free portion of the mesentery as described before (8, 9). The laser lesion induced a lasting vasoconstruction of about 10%. The diameter of the laser shot is about 5 μm. There is relatively wide range of flow pattern which has little influence on the rate of thrombus formation unless flow is markedly decreased. Thrombus formation was evaluated by direct observation through the microscope.

For the evaluation of thrombus formation the number of laser injuries required to induce a defined thrombus was used. Laser shots were repeated at 1 min intervals until a thrombus had formed, which was at least as long, and as broad as the vessel diameter. An increased number of laser injuries needed to induce this defined thrombus corresponds with an antithrombotic effect. If possible, five animals per drug dose were investigated, and in each animal three different vessels were injured. Control animals were investigated daily. They were selected at random. For statistical evaluation the Kruskal-Wallis test has been used (10, 11).

RESULTS

In vitro effects of Na-PPS and Ca-PPS

All two pentosan polysulphate showed similar anticoagulant activity in pooled rat plasma (*Table 1*). APTT was inhibited in concentrations 5 μg/ml and Heptest in concentration 10 μg/ml. Heptest was prolonged 1,5-fold at concentrations 25 μg/ml of Na-PPS in comparison to same of Ca-PPS.

Table 1. Effect of Na-PPS and Ca-PPs on aPTT and Heptest in rat PTT. Rat pool plasma, n-3.

DOSE mg/ml	aPTT		HEPTEST	
	Na-PPS	Ca-PPS	Na-PPS	Ca-PPS
0.025	> 120 *	> 120 *	410.8 ± 62.8 *	284.1 ± 71.2 *
0.01	79.1 ± 8.4 *	83.2 ± 10.1 *	87.2 ± 6.1 *	92.4 ± 8.4 *
0.005	49.3 ± 9.1 *	48.6 ± 8.7 *	59.6 ± 14.7	69.2 ± 3.2
0.001	28.1 ± 4.6	27.4 ± 5.9	71.2 ± 3.1	68.1 ± 4.3
CONTROL	28.4 ± 2.6		68.2 ± 3.7	

* $p < 0.05$

Ex vivo anticoagulant effects in rats

The *ex vivo* results obtained 120—140 min after subcutaneous administration of different doses of Na-PPS and Ca-PPS are summarized in *Table 2*. Blood samples were obtained after the investigations in the laser model. After subcutaneous injection of investigated agents, aPTT as well as Heptest were markedly prolonged. Na-PPS and Ca-PPS above 10 mg/kg led to prolongation 3-fold of aPTT and 2.5-fold of Heptest compared to the results obtained with control.

After oral application of Na-PPS and Ca-PPS in investigated doses up to 50 mg/kg clotting time was not prolonged.

Table 2. Ex vivo studies of aPTT and Heptest performed in rats 2 h after s.c. injection of different doses of Na-PPS and Ca-PPS, n-5.

DOSE mg/ml	aPTT		HEPTEST	
	Na-PPS	Ca-PPS	Na-PPS	Ca-PPS
20.0	> 120 *	114.1 ± 21.6	> 600 *	> 600 *
10.0	79.4 ± 12.6 *	87.4 ± 16.1 *	154.2 ± 31.6 *	194.1 ± 37.6 *
5.0	28.2 ± 5.2	31.2 ± 6.4	61.4 ± 12.1	73.8 ± 10.2
CONTROL	28.1 ± 2.6		67.2 ± 4.9	

* $p < 0.05$

Inhibition of thrombus formation in the laser model

In the animal model investigated agents showed a significant and dose-dependent antithrombotic effect. The results obtained in the laser model

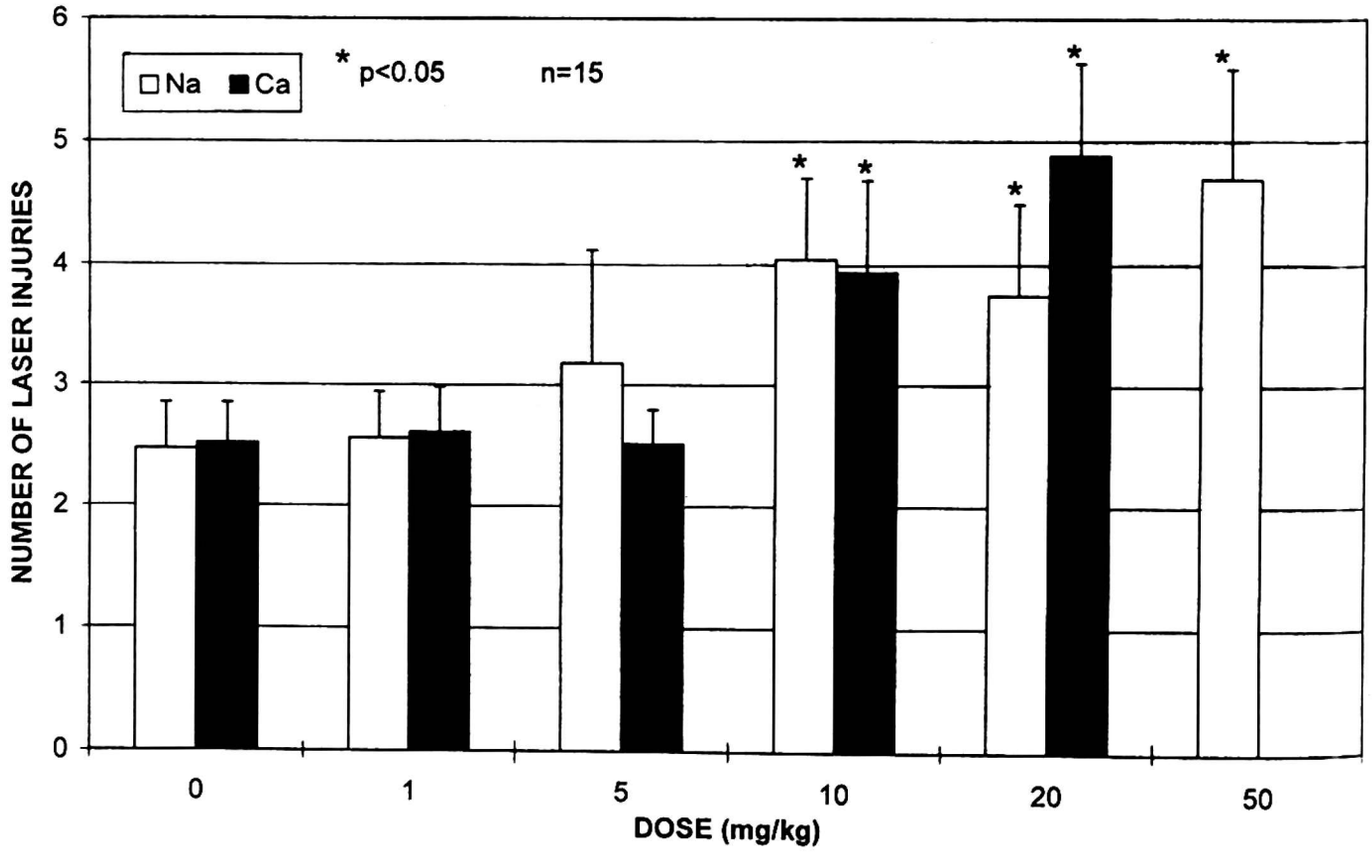

Fig. 1. Antithrombotic effect of different doses of sodium and calcium PPS investigated 2 h after subcutaneous injection; venules. Number of laser injuries needed to induce a defined thrombus. (Mean ± SD).

with control rats and after subcutaneous injection of investigated agents are summarized in *Fig. 1*. In control animals, 2,38 ± 0,42 and 2,52 ± 0,38 laser injuries were necessary to induce the formation of thrombus. Injection of Na-PPS and Ca-PPS in-dose 10 mg/kg and more led to an increase of the number of the laser injuries. The subcutaneous injection of 10 mg/kg Na-PPS significantly inhibited thrombus formation after 2,4 and 8 h. After 12 h, no significant effect was present (*Fig. 2*). The antithrombitic effect lasted 12 h for Ca-PPS. After oral administration of Na-PPS in doses up to 50 mg/kg antithrombotic effect was not observed (*Fig. 3*).

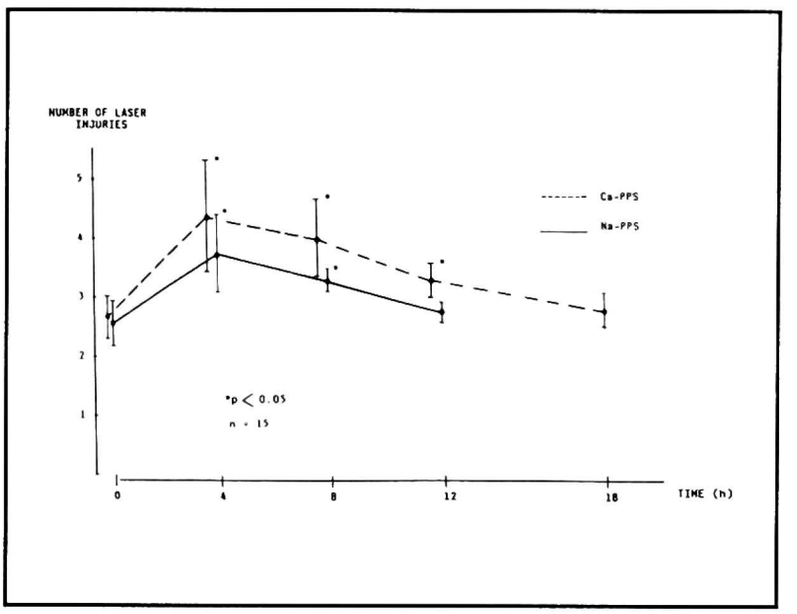

Fig. 2. Duration of antithrombotic effect of sodium PPS 10 mg/kg s.c. and calcium PPS 10 mg/kg s.c.; venules. Number of laser injuries needed to induce a defined thrombus. (Mean ± SD).

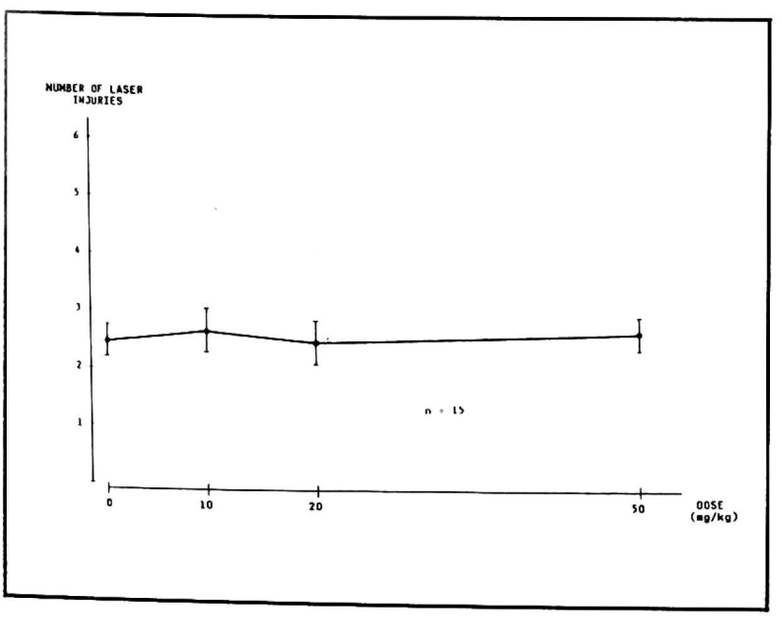

Fig. 3. Sodium PPS 2 h after oral administration; venules. Number of laser injuries needed to induce a defined thrombus. (Mean ± SD).

Oral application of Ca-PPS in doses 20 mg/kg and higher significantly inhibited thrombus formation (*Fig. 4*).

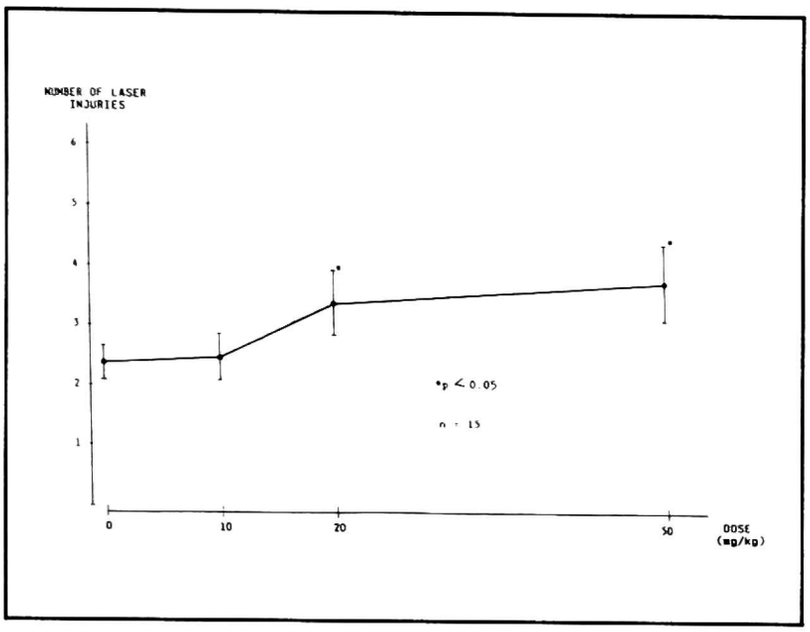

Fig. 4. Effects of calcium PPS in the laser model 2 h after oral application; venules. Number of laser injuries needed to induce a defined thrombus. (Mean ± SD).

DISCUSSION

Different models of experimental thrombosis have been developed. Microthrombosis induced by laser beam has the greatest resemblance to the pathological processes resulting from a lesion observed in human disorders. Indeed, with this model, it was possible to obtain a restricted destruction of the endothelial wall (12). We have used this model to study the antithrombotic effect of the different antithrombotic agents alone and in combination. In this experimental model stable PGI_2 — analogue Cicaprost, thromboxane receptor antagonists Daltroban and HN-11501, Aspisol, low molecular weight heparins and unfractionated heparin effectively impaired thrombus formation in a dose-dependent manner (13, 14). Defibrotide, a natural polydeoxyribonucleotide, Molsidomin and Aprosulfate (bis lactobionic acid amide) also were quite effective in the laser model. The strongest additive inhibiting effect on thrombus formation was observed after the administration of minimal effective doses of Molsidomin together with Cicaprost and Defibrotide (15, 16).

In the present study we investigated anticoagulant and antithrombotic effects of sodium and calcium derivates of pentosan polysulphate. Sodium and calcium PPS showed similar anticoagulant activity in pooled rat plasma. They markedly prolonged clotting time (aPTT, Heptest) *in vitro*.

Wagenvoord *et al.* (1) recently demonstrated that PPS inhibits on the level of factor VIII, and factor V activation by thrombin also can be inhibited, however about 30-fold higher concentrations of PPS are required to obtain the same effect. On basis of the results of authors that PPS mainly acts on factor VIIIa activation, it can predict that PPS should have an effect on the aPTT.

After subcutaneous injection of investigated agents aPTT as well as Heptest were prolonged at concentration above of 10 mg/kg sodium and calcium PPS. Pentosan polysulphate has a marked effect on the intrinsic generation of Xa (17). The effect of PPS on the extrinsic blood coagulation should be less than on the intrinsic blood coagulation (1). Vinazzer *et al.* (18) also found a significant effect on Xa generation, and claimed that the effect of PPS was comparable to that of heparin, especially after subcutaneous injection. Pentosan polysulphate a heparin related substance, is able to release *in vivo* at therapeutic concentrations a significant anti-Xa activity not releated to AT III (17). A similar Xa inhibition was described and associated to HTGL (Hepatic trigliceride lipase), therefore it was suggest that PPS might exert its anticoagulant effect through release of this lipase (19). In contrast, Ofosu *et al.* (20) reported that PPS which is relatively poor potentiator of factor Xa inactivation, was a very effective inhibitor of thrombin generation. This concept is supported by recent *in vivo* studies using pentosan polysulphate, which show that this agent, which has predominantly anti-thrombin activity, is effective antithrombotic agent (21).

The relevance of both anti-Xa and anti-IIa activities to antithrombotic properties has been the subject of much current debate (22—25), but there is general agreement in that their levels are not in parallel with the antithrombotic efficacy. The formation of microthrombi in small mesenteric venules after laser injuries was significantly reduced after administration of adequate doses of the investigated PPS. Sodium and calcium PPS significantly inhibited thrombus formation in dose 10 mg/kg s.c. This effect was more pronounced after application of 20 and 50 mg/kg. This finding is in accordance with the results obtained by several other investigators (3, 26) who also reported that PPS inhibited experimental thrombosis.

The duration of antithrombotic effects lasted 8 h for sodium-PPS and 12 h for calcium-PPS. This long-lasting activity may be caused slow releasing of PPS from subcatoneous tissue. Endothelial banding of the PPS and the inhibition of thrombus at the endothelial side could also possible explain this long duration of the antithrombotic actions. After oral application of 50 mg/kg sodium and calcium PPS, clotting time was not prolonged, whereas oral application of calcium PPS in doses higher than 20 mg/kg significantly inhibited thrombus formation. In the observation of Klöcking *et al.* (27) determination of the partial thromboplastin time (aPTT) served to establish the anticoagulant effect after intravenous and oral administration of 5 mg/kg. The aPTT values were significantly prolonged up to 4 h after application. Losonczy *et al.* (28) suggested that PPS is an efective drug for increasing fibrinolysis by oral administration. Doses of approximately 200 mg may be recommended after individual testing of patients with thromboembolism and impaired fibrinolysis. In conclusion, results of our study indicate that calcium-PPS seems to have

a slightly longer lasting antithrombotic effect than sodium-PPS. With calcium-PPS an antithrombotic effect was observed after oral application of relatively high doses. This direct action probably is due releasing of tissue plasminogen activator.

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